REMARKS

Claims 1-11, 13-17, 19-24, 26-28, 35-37, 41, 44, 47, and 50 are pending. Non-elected claims 12, 18, 25, 29-32, 34, 39, 42-43, 45, 48, and 51-52 are withdrawn from further consideration by the Examiner, and thus have been cancelled without prejudice or disclaimer. Claims 1-11, 13-17, 19-24, 26-28, 35-37, 41, 44, 47, and 50 have been rejected on various grounds.

In this response, applicants amend claims 1, 4, 10, 11, 16, 21, 28, 35, 36 and provided the arguments as set forth below. Reconsideration and allowance are requested in view of the amendments and below arguments.

Specification

Page 3, item 5 of the Office Action: Applicants have amended specification following the Examiner's suggestion.

Page 3, item 6 of the Office Action: Applicants add an "abstract of the disclosure" as published under WO 97/02454, which does not constitute "a new matter".

Page 3, items 7 and 8 of the Office Action: Applicants have amended the specification to overcome the informalities objection.

Claim Objections

On page 4, item 9 of the Office Action the Examiner has objected to claims 10 and 36 because of informalities. In response, Applicants amend the claims 10 and 36, per Examiner's suggestion, to correct typographical errors, and as appropriate.

Written Description Rejections

On pages 4-5, items 10-11 of the Office Action the Examiner has rejected claims 1-11, 13-17, 19-20, 23, 26-28, 35-37, 41, 44 and 47 under 35 U.S.C 112, first paragraph. The Examiner asserts that the specification does not reasonably provide a written description of "*any* soluble derivative of *any* soluble polypeptide" comprised of *any* of the various elements of the targeting constructs (these membrane binding elements will be referred to hereinafter as "sequential membrane addressins or SMAs"). Applicants respectfully disagree with the Examiner.

The USPTO has issued its final guidelines for written description (66 Fed. Reg. 1099). The written description guidelines first instruct examiners to determine what the claim as a whole covers and then review the entire specification to determine whether all subject matter that is essential to the invention is actually recited in the claims. See written description guidelines at II(A)(1), (2). Next, the examiners are instructed to determine whether the applicant was in possession of all that is claimed. See the written description guidelines at II(A)(3). According to the guidelines, possession of a claimed invention can be shown by disclosure of structural characteristics, functional characteristics that correlate with structure or combinations thereof. See the written description guidelines at II(A)(3)(a). Claims that encompass a genus must be supported by a written description of a representative number of species. See the written description guidelines at II(A)(3)(a)(2). The written description of the representative species of the genus can be shown by disclosure of structural characteristics, functional characteristics that correlate with structure or combinations thereof. Applicants submit that the Examiner has not satisfied these guidelines in making the rejection, which alone is grounds for withdrawal of the rejection.

in the specification. Applicants point out that the table of therapeutic proteins on page 5 of the specification lists a selection of "soluble polypeptides" with known therapeutic properties. Examples of three of these categories (complement inhibitors, thrombolytics and antibodies) can be found in the specification (see Examples 8, 12, 13, and 15, 16, 17 in the specification). The soluble polypeptides described in these examples cover several wide-ranging categories of proteins.

Applicants submit that it is clear that they had possession of the subject matter claimed in claims 1-11, 13-17, 19-20, 23, 26-28, 35-37, 41, 44 and 47. Given the correspondence and applicants' identification of this correspondence, a heavy burden is placed upon the Examiner to reject the claims. *See* MPEP § 2163.04.

Definiteness Rejections

On page 6, items 12-13 of the Office Action the Examiner has rejected claims 1-11, 13-17, 19-20, 23, 26-28, 35-37, 41, 44 and 47 under 35 U.S.C 112, second paragraph. Applicants respectfully disagree with the Examiner and refers to the following:

For definiteness, a claim need only reasonably apprise those skilled in the art of the utilization and scope of the invention. *Hybritech, Inc. v. Monoclonal Antibodies,* 231 USPQ 81, 94-95 (1986). Words are to be given their plain meaning as understood by the person of ordinary skill in the art, particularly given the limitations of the English language. *See* MPEP §§ 707.07(g); 2111.01. Claims are to be given their broadest reasonable interpretation consistent with applicants' specification. *See* MPEP § 2111. In sum, in order to reject the claims on definiteness grounds, it is incumbent on the examiner to show how and why the skilled person having applicants' specification

would not be apprised of the invention by the language-at-issue. The rejections are discussed below.

The Examiner has rejected claims 1 for using the term "heterologous" in conjunction with the phrase "not all identical". Applicants amend claim 1 by deleting the phrase "heterologous" to avoid alleged ambiguity.

The Examiner has rejected claims 1 for using the term "thermodynamic additivity". In response, Applicants respectfully disagree with the Examiner and point out that "thermodynamic additivity" is well known in the art. Applicants provided herewith several related references on "additivity" [see **EXHIBIT-1**]. Applicants further states that the term "thermodynamic additivity" means that if one binding process is characterized by a given change in free energy and a second such process has another free energy change, then combining these processes in one molecule will result in a new binding process characterized by a third free energy which approximates the *sum* of the first two. In terms of dissociation constants, this approximates the *product* of dissociation constants for the separate processes. The additivity referred to is therefore the addition of hydrophobic and electrostatic binding free energies which results from independent binding processes mediated by the individual binding elements or SMAs

The Examiner has rejected claim 4 for using the term "for specific membranes". In response, Applicants state that the term "for specific membranes" refers to "cell membranes" for example, outer cell membranes, of specific cell types (for example, red blood cells, endothelial cells, epithelial cells, spermatozoa, fibroblasts etc.), each of which has different structural characteristics which may interact differently with membrane-binding elements or SMAs. Nevertheless, Applicants have amended the claim

The Examiner has objected to the use of the term "N-terminus on left" in claims "10 and 48", we think the Examiner in fact meant claims "11 and 21", wherein the term is recited. Applicant amend the claim deleting the term from the claims.

The Examiner states that the recitation of the terms "a flexible linker group in claim 14" and "linker group" in claim 15 are ambiguous. In response, Applicants respectfully disagree and provide the following argument: When a "linker" is used, the membrane binding elements would still be linked "covalently". Those skilled in the art would understand that elements could be either spaced apart or linked together directly, subject to any requirements, and they would have the knowledge to make such basic linkers, if desired. Therefore, recitation of the terms "a flexible linker group in claim 14" and "linker group" in claim 15 are not ambiguous. Allowance of claims are therefore solicited.

Applicants amend claim 16 to be dependent on claim 15, wherein the term "bridging group" is recited.

Applicants amend the claim 28 as suggested by the Examiner.

The Examiner has rejected the claim 35 for using the phrase "(optionally C-substitutes)". In response the Applicants provide the following arguments: The claim refers to a derivative of a fatty acid binding element, for example, a C_{6-20} fatty acyl derivative of an amino C_{2-6} alkane thiol, wherein the alkane thiol can be substituted. Two examples of such derivatives are mentioned in the specification (see the bridging paragraph on pages 16-17 of specification). The second, N-myristoyl L-cysteine is an example of a C-substituted fatty acid derivative. Examples of generic derivatives (I) and the cysteine derivative (II) are shown in **EXHIBIT-2.** For further clarity, Applicants

Applicants amend claim 36 and made properly dependent on claim 35.

Applicants further point out that the amended claims are clear and understandable with respect to the terms and phrases used. Therefore, withdrawal of the rejections is solicited.

Anticipation Rejections

On pages 7-9, items 14-16 of the Office Action, the Examiner has rejected the claims 1-3, 5-11, 14-15 and 26 and alleged as being anticipated by Sigal *et al.* and U.S. Patent No. 5,776,689 for reasons recorded by the Examiner in Paper No. 13. Applicants respectfully disagree with the Examiner and note that:

In order to reject a claim under 35 USC § 102, the examiner must demonstrate that each and every claim term is contained in a single prior art reference. See Scripps Clinic & Research Foundation v. Genentech, Inc., 18 USPQ2d 1001, 1010 (Fed. Cir. 1991); Hybritech, Inc. v. Monoclonal Antibodies, Inc., 231 USPQ 81, 90 (Fed. Cir. 1986); see also MPEP § 2131. Claim terms are to be given their plain meaning as understood by the person of ordinary skill in the art, particularly given the limitations of the English language. See MPEP §§ 707.07(g); 2111.01. Claims are to be given their broadest reasonable interpretation consistent with applicants' specification. See In re Zletz, 13 USPQ2d 1320, 1322 (Fed Cir. 1989) (holding that claims must be interpreted as broadly as their terms reasonably allow); MPEP § 2111.

Not only must the claim terms, as reasonably interpreted, be present, an allegedly anticipatory reference must enable the person of ordinary skill to practice the invention as claimed. Otherwise, the invention cannot be said to have been already within the public's possession, which is required for anticipation. See Akzo, N.V. v.

U.S.I.T.C., 1 USPQ2d 1241, 1245 (Fed. Cir. 1986); In re Brown, 141 USPQ 245, 249 (CCPA 1964).

Applicants provides the following arguments to obviate alleged 35 USC § 102 rejections:

(a) Sigal *et al.* describes the phenomenon of thermodynamic additivity between hydrophobic and electrostatic interactions mediated by myristoyl and basic peptide residues in the protein Src. Sigal *et al.* suggested (p. 12255 col 1) that the role of the N-terminal basic residues in Src might not be to interact with a specific receptor as had been previously suggested but might be to reduce the electrostatic potential at the surface of vesicles. Sigal *et al.* used synthetic peptides to compete for Src binding to vesicles but they did not employ such peptides to mediate such binding in soluble proteins. There is no teaching in Sigal *et al.* of any such process of post-translational modification or any suggestion of how to achieve it in practice or of its possible utility. Applicants point out to note that Src is an intracellular protein which does not normally exist in a soluble form outside the cell and that the application of modification with myristoylated basic peptides to soluble extracellular proteins or the potential generality of such an approach could not be deduced from the data described by Sigal *et al.*

Applicants further point out that the Claim 1 of the application relates to polypeptide derivatives comprising two or more membrane binding elements that are capable of interacting with "components of cellular or artificial membranes exposed to extracellular fluids" (emphasis added) *e.g.*, to the external surface of a cell. Sigal *et al.* studied the internal membrane binding of Src. Sigal *et al.* on page 12253, column 1, described the transcription of the internal peptide. On page 12253, column 2, line 3, Sigal *et al.* described the association of the myristoyl with the interior of the lipid bilayer.

peptide. For example, Kaplan *et al.* (Mol. Cell. Biol. 1990. 10, 1000-1009. Cited as reference 6 on page 12257 of Sigal *et al.*) describe the various internal cellular structures that the amino-terminal portions of the Src protein bind (see **EXHIBIT-3**: 'abstract' on page 1000 of Kaplan *et al.*); Buss *et al.* (Mol. Cell. Biol. 1984, 4, 2697-2704 cited as reference 7 on page 12257 of Sigal *et al.*) state that Src "appears at the cytoplasmic face of the plasma membrane" (see **EXHIBIT-4**).

Hence, after reading Sigal *et al.* and the many of the references cited in Sigal *et al.*, it is clear that Src is targeted to internal membranes. There is no incentive to modify an extracellular peptide with a membrane targeting motif from Src as this motif clearly targets Src to an internal surface and not to the external surface of a cell.

Therefore Sigal *et al.* do not anticipate the current invention.

(b) Karin *et al.* (the '689 patent) describes a protein recruitment system in which a protein-protein interaction is detected by recruitment of an effector protein to an intracellular compartment. This is achieved by the use of the protein Sos which is capable of translocation to the inner plasma membrane of a cell where it interacts with and activates another protein Ras. This translocation is mediated in both Ras and Sos by the "myristoyl-electrostatic switch" in which the membrane binding function is activated or deactivated by reversible phosphorylation of the basic peptide domain. The Ras/Sos interaction can be used to detect interactions between other intracellular proteins in solution by expressing fusion proteins involving those other interacting agents. Such fusion proteins can be engineered to contain a localization domain comprising a myristoylation signal and the basic effector sequence – expression of such proteins is dependent on the myristoylation machinery of the cell and is restricted to the intracellular compartment. The '689 patent does not teach how to obtain fusion or other

such constructs, if produced, would bind to the outer cell surface. The '689 patent provides for a cellular detection system which yields a method for identifying protein-protein interactions; it does not provide for modified proteins of therapeutic utility.

Although the '689 patent describes a fusion protein consisting of two membrane binding elements, like Sigal *et al.*, again, these are internal binding elements. Even with such references there would still be no incentive to add these to an extracellular protein to bind this protein to the extracellular membrane. This citation does not anticipate the current invention.

Therefore Sigal *et al.* and the '689 patent do not anticipate the claimed invention of the instant application. Withdrawal of rejections and allowance of claims 1-3, 5-11, 14-15 and 26 is solicited.

Obviousness Rejections

On pages 9-13, items 17-21 of the Office Action, the Examiner has rejected claims 1, 13-17, 19-24, 37, 41, 44, and 50 as obvious over Sigal *et al.* in combination with US patent No. 5,472,939; or EP 0207589, or EP 0155387, or US patent No. 5,326,700; or EP 0152736. In response the Applicants respectfully traverse the rejection and refer the arguments of the above paragraphs made in order to obviate the alleged §102 rejections. In view of the above argument, the Applicants point out that Sigal *et al.* do not rectify the deficiencies in US patent No. 5,472,939 (the '939 patent); or EP 0207589 (the '589 patent), or EP 0155387 (the '387 patent), or US patent No. 5,326,700 (the '700 patent); or EP 0152736 (the '736 patent).

At the outset, Applicants note the examiner must show all of the recited claim elements in the combination of references that make up the rejection. When combining

show by citation to specific evidence in the cited references that (i) there was a suggestion to make the combination and (ii) there was a reasonable expectation that the combination would succeed. Both the suggestion and reasonable expectation must be found within the prior art, and not be gleaned from applicants' disclosure. *In re Vaeck*, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991); *In re Dow Chemical Co.*, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988); *see also* MPEP §§ 2142-43.

When an examiner alleges a *prima facie* case of obviousness, such an allegation can be overcome by showing that (i) there are elements not contained in the references or within the general skill in the art, (ii) the combination is improper (for example, there is a teaching away or no reasonable expectation of success) and/or (iii) objective indicia of patentability exist (for example, unexpected results). *See U.S. v. Adams*, 383 U.S. 39, 51-52 (1966); *Gillette Co. v. S.C. Johnson & Son, Inc.*, 16 USPQ2d 1923, 1927 (Fed. Cir. 1990); *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve*, 230 USPQ 416, 419-20 (Fed. Cir. 1986). Applicants submit that the rejections do not meet this test.

Applicants further explain that:

(a) Fearon *et al.* (the '939 patent) patent described the CR1 gene and protein and methods for treating complement-mediated disorders based upon of the use of soluble forms of CR1, which lack the transmembrane domain. The reason for the use of soluble complement regulatory proteins is that, unlike the intact CR1 molecule, they can be secreted from recombinant host cells in active forms and can be made in bulk as pharmaceuticals. While the '939 patent describes the potential use of such pharmaceutical preparations in medicine, it certainly does not teach that such materials could be modified to *restore* the ability to bind to membranes (characteristic of native CR1 but not the soluble fragments) or that such membrane binding would have a

asserted that it would have been obvious to one of ordinary skill in the art at the time invention was made to combine the binding elements identified by Sigal *et al.* with a soluble form of CR1, but the Examiner provides no explanation of motivation. To the contrary, at the time the invention was made, there was no evidence that conferring a means of binding to the *inner* membrane leaflet (as described in Sigal *et al.*) would be of any value for CR1 (which is present on the *outer* leaflet of cells) and acts on extracellular complement activation either there or (as a soluble form) in extracellular bulk solution (as described in Fearon *et al.*). Furthermore, the large increases in potency observed when applying the modifications of the present invention were quite unexpected.

(b) Robinson *et al.* (the '589 patent and the '387 patent), Ferres *et al.* (the '736 patent) and Berg *et al.* (the '700 patent): These patents describe various derivatives and analogs of plasminogen activators for use as fibrinolytic agents. The primary objective of the modifications employed was either to increase the biological half-life of the fibrinolytic agent or, in the case of the '700 patent, to improve the production of one such agent (t-PA). The utility of thrombolytic agents as a drug class is emphasised by these cases but none of them teach that directing a fibrinolytic agent to a cell membrane would be useful in the treatment of thrombosis nor do they suggest a means of achieving this end. At the time that the current invention was made, improvements to thrombolytic agents were focused on either increasing the plasma half-life or on improving the potency and selectivity of these enzymes. It was not obvious that attachment of the peptides of Sigal *et al.* would achieve those objectives and, in fact, directing fibrinolytics to cell surfaces *in vivo* would actually tend to *decrease* the plasma half-life by providing a new clearance pathway. Those skilled in the thrombolysis art would therefore not have been motivated to use the membrane-directing modification.

A general point about the obviousness and utility of the instant invention should also be made. The invention provides a practical means for solving a generic problem in biotechnology. Where gene products are stably anchored in cells (as in the case of CR1), the only way to exploit the therapeutic potential of their biological activities is to overexpress them in secreted truncated forms. Examples of this approach are well documented (such as the use of truncated TNF receptors to sequester TNF). However, this approach frequently gives a product, which has low biological activity because the latter is manifest best in the original structural context of the membrane protein (*i.e.* in the membrane). By providing a means to generate *soluble* but *membrane-binding* derivatives of such proteins, the present invention allows them to be exploited as drugs. This important utility is not anticipated by any of the references cited above.

Thus, at the time the invention was made, there would have been no "reasonable expectation of success," to produce the soluble derivatives of soluble polypeptides of the current invention, even while having the knowledge of Sigal *et al.* and in combination with the disclosure of US patent No. 5,472,939; or EP 0207589, or EP 0155387, or US patent No. 5,326,700; or EP 0152736. Applicants therefore submit that the Examiner has not established a *prima facie* case of obviousness, and therefore respectfully request withdrawal of the rejections.

CONCLUSION

In view of the foregoing remarks and amendments, reconsideration of the application and allowance of the claims are requested. If any issues remain which the Examiner believes could be resolved through a Supplemental Response or an Examiner's Amendment, the Examiner is respectfully requested to contact the undersigned at 202-912-2777.

Respectfully submitted,

Date: January 28, 2002

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MARKED-UP AMENDED CLAIMS

1. **(once amended)** A soluble derivative of a soluble polypeptide, said derivative comprising two or more **[heterologous]** membrane binding elements with low membrane affinity covalently associated with the polypeptide, which elements are not all identical and are capable of interacting, independently and with thermodynamic additivity, with components of cellular or artificial membranes exposed to extracellular fluids.

4. **(twice amended)** A derivative according to claim 1 which incorporates sufficient elements with low affinities for membrane components to result in a 0.01-10nM dissociation constant affinity for specific **cell** membranes.

10. **(twice amended)** A derivative according to claim 8 wherein a membrane binding element is a basic amino acid sequence including $(Lys)n$, where n is from 3 to 10.

11. **(once amended)** A derivative according to claim 10 wherein the amino acid sequence is selected from **the group consisting of**:

i) DGPKKKKKKSPSKSSG (SEQ ID No. 37),
ii) GSSKSPSKKKKKKPGD (SEQ ID No. 39),
iii) SPSNETPKKKKKRFSFKKSSG (SEQ ID No. 41),
iv) DGPKKKKKKSPSKSSK (SEQ ID No. 43), **and**
v) SKDGKKKKKKSKTK (SEQ ID No. 45).

16. **(twice amended)** A derivative according to claim **[14] 15** wherein the chemical bridging groups are of formula (I):

-A-R-B- (I)

in which each of A and B, which may be the same or different, represents -CO-, -C(=NH 2^+)-, maleimido, -S- or a bond and R is a bond or a linking group containing one or more -(CH_2)-or meta-ortho- or para-disubstituted phenyl units optionally together with a hydrophilic portion.

21. **(once amended)** A soluble derivative according to claim 20 wherein the soluble CR1 polypeptide consists of residues 1-196 of CR1 and with an N-terminal methionine and the derivative comprises a myristoyl group and one or more polypeptides sequence selected from **the group consisting of:**

i) DGPKKKKKKSPSKSSGC,

ii) GSSKSPSKKKKKKPGDC,

iii) CDGPKKKKKKSPSKSSK,

iv) SKDGKKKKKKSKTKC,

v) CSAAPSSGFRILLLKV,

vi) AAPSVIGFRILLLKVAGC, and

vii) DGPSEILRGDFSSC

[(N-terminus on left)].

28. **(once amended) A polypeptide portion of a derivative according to claim 1, wherein [T]**the polypeptide **is selected from the group consisting** of SEQ

35. **(twice amended)** A soluble derivative of a soluble polypeptide according to claim 1, wherein at least one membrane binding element with low membrane affinity is a C_{10-20} fatty acyl derivative of an aminoC_{2-6} alkane thiol **[(optionally C-substituted)], wherein the C in alkane thiol is optionally substituted**.

36. **(once amended) The soluble derivative of [A compound according to]** claim 35 **is** selected from **the group consisting of** N-(2-myristoyl) aminoethanethiol **a**nd N–myristoyl L-cysteine.

EXHIBIT -1:

Thermodynamic Additivity References:

Decomposition of the free energy of a system in terms of specific interactions. Implications for theoretical and experimental studies.

J Mol Biol 1994 Jul 8;240(2):167-76

Mark AE, van Gunsteren WF.

Department of Physical Chemistry, Swiss Federal Institute of Technology, ETH Zentrum, Zuerich.

Recently, a number of methods have been proposed that are designed to extract contributions to the change in free energy associated with a given perturbation or mutation of a protein originating from specific residue-residue or atom-atom interactions, both based on theoretical calculations and on experimental data. We caution here that detailed analysis based on these methods is unreliable. It is demonstrated, both from first principles using statistical mechanics and by way of example, that in a general case a meaningful decomposition of the free energy in terms of specific residue-residue or atom-atom interactions is not possible.

Water molecules participate in proteinase-inhibitor interactions: crystal structures of Leu18, Ala18, and Gly18 variants of turkey ovomucoid inhibitor third domain complexed with Streptomyces griseus proteinase B.

Protein Sci 1995 Oct;4(10):1985-97: Huang K, Lu W, Anderson S, Laskowski M Jr, James MN.

Department of Biochemistry, University of Alberta, Edmonton, Canada.

Crystal structures of the complexes of Streptomyces griseus proteinase B (SGPB) with three P1 variants of turkey ovomucoid inhibitor third domain (OMTKY3), Leu18, Ala18, and Gly18, have been determined and refined to high resolution. Comparisons among these structures and of each with native, uncomplexed SGPB reveal that each complex features a unique solvent structure in the S1 binding pocket. The number and relative positions of water molecules bound in the S1 binding pocket vary according to the size of the side chain of the P1 residue. Water molecules in the S1 binding pocket of SGPB are redistributed in response to the complex formation, probably to optimize hydrogen bonds between the enzyme and the inhibitor. There are extensive water-mediated hydrogen bonds at the interfaces of the complexes. In all complexes, Asp 26 of

surface area buried in the interface of the corresponding complexes. The resulting constant of proportionality is 34.1 cal mol-1 A-2. These structures confirm that the binding of OMTKY3 to the preformed S1 pocket in SGPB involves no substantial structural disturbances that commonly occur in the site-directed mutagenesis studies of interior residues in other proteins, thus providing one of the most reliable assessments of the contribution of the hydrophobic effect to protein-complex stability.

Protein-protein interactions: additivity of the free energies of association of amino acid residues.

J Theor Biol 1985 Sep 7;116(1):149-59: Horovitz A, Rigbi M.

Additivity of contributions to the free energies of association of ovomucoid third domain inhibitors with elastase, chymotrypsin and subtilisin (Laskowski, 1980; Laskowski *et al.*, 1981, 1983; Empie & Laskowski, 1982) is fully demonstrated by applying the mathematical method of Free and Wilson (1964) for calculating the effects of substitutions in a family of drugs. Also demonstrated is the ability to predict the activity of third domain sequences. Sensitive regions in the contact area of inhibitor and enzyme are mapped, and a sequence is suggested for a new, more powerful and selective ovomucoid third domain inhibitor of subtilisin.

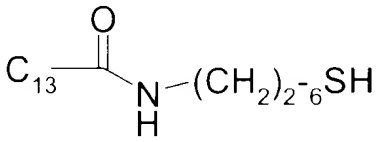
Measures of cooperativity in the binding of ligands to proteins and their relation to non-additivity in protein-protein interactions.

Proc R Soc Lond B Biol Sci 1986 Dec 22;229(1256):315-29: Horovitz A.

The analogy between cooperativity in the binding of ligands to proteins and non-additivity in protein-protein interactions is demonstrated and discussed in terms of the Wong and the Hill coefficients. A measure of non-additivity, the interaction constant, is rigorously derived for four thermodynamic cycles, involving the binding of small molecules to proteins and protein association. It is the reciprocal of the 'defect factor' of Laskowski *et al.* in Proteinase inhibitors: medical and biological aspects (ed. N. Katunuma *et al.*), pp. 55-68 (1983), and its logarithm is the Wong measure of cooperativity. These three measures are thus here given a common theoretical basis. The Hill coefficient for an asymmetric dimer that binds two different ligands which do not compete for the same site, at 50% saturation of each site, is derived. It is shown to be a function of the interaction constant and of the fraction of protein to which ligand is bound at both sites. These relations for protein-ligand interactions are then discussed in the context of non-additivity in protein-protein interactions.

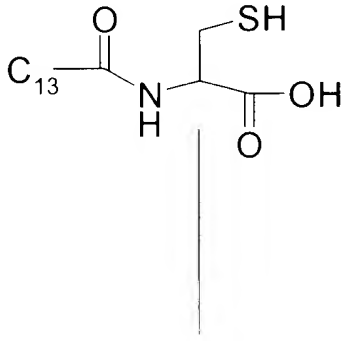
EXHIBIT-2:

Membrane Binding Elements may be Fatty Acid Derivatives



(I)

Myristoyl derivative of an amino C2-6 alkane thiol



C-substituted group

(II)

Myristoyl L-cysteine (A C-substituted alkane thiol)

EXHIBIT-3:

Kaplan *et al.* Mol. Cell. Biol. 1990. **10:**1000-1009.

MOLECULAR AND CELLULAR BIOLOGY, Mar. 1990, p. 1000–1009
0270-7306/90/031000-10$02.00/0

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The *src* Protein Contains Multiple Domains for Specific Attachment to Membranes

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The proteins encoded by the oncogene v-*src* and its cellular counterpart c-*src* (designated generically here as pp60^{src}) are tightly associated with both plasma membranes and intracellular membranes. This association is due in part to the amino-terminal myristylation of pp60^{src}, but several lines of evidence suggest that amino-terminal portions of the protein itself are also involved. We now report that pp60^{src} contains at least three domains which, in conjunction with myristylation, are capable of mediating attachment to membranes and determining subcellular localization. We identified these domains by fusing various portions of pp60^{src} to pyruvate kinase, which is normally a cytoplasmic protein. Amino acids 1 to 14 of pp60^{src} are sufficient to mediate both myristylation and the attachment of pyruvate kinase to cytoplasmic granules. In contrast, amino acids 38 to 111 mediate association with the plasma membrane and perinuclear membranes, whereas amino acids 204 to 259 mediate association primarily with perinuclear membranes. We conclude that pp60^{src} contains independent domains that target the protein to distinctive subcellular locations and thus may facilitate diverse biological functions of the protein.

The products of the retroviral oncogene v-*src* and its cellular counterpart c-*src* (designated generically here as pp60^{src}) are protein tyrosine kinases that are associated with membranes. Association of pp60^{src} with membranes is essential for transformation by v-*src* (19). In various cell types, the viral and cellular *src* proteins are associated with plasma membranes (7, 8, 21, 44), with perinuclear membranes (36), with secretory organelles in both chromaffin cells and platelets (30, 33), and with growth cones in developing neurons (27, 41). Little is known about how pp60^{src} is specifically targeted to these subcellular locations.

The amino terminus of pp60^{src} is covalently coupled to a 14-carbon fatty acid, myristic acid (4, 39). The myristyl moiety promotes the association of pp60^{src} with membranes (2, 11). On the other hand, not all myristylated *src* proteins are associated with membranes (3, 11), and some nonmyristylated *src* proteins are membrane associated, albeit weakly (12, 20). These results suggest that pp60^{src} contains amino acid sequences which, in conjunction with amino-terminal myristylation, mediate the association of the protein with membranes.

Previous work has implicated the amino-terminal 10 kilodaltons (kDa) of pp60^{src} in attachment to membranes (22, 35). Here we report work that dissects the amino-terminal domain into several regions that independently target and attach the protein to membranes in distinctive subcellular locations.

MATERIALS AND METHODS

Materials. [illegible] and RL18PK12X, which differ by the reading frame at a [illegible] polylinker sequence), from B. Roberts (16); and a plasmid containing the chicken fibroblast c-*src* cDNA, p5H, from H. Hanafusa. The E3 *src* gene is a deletion mutant which lacks the sequence encoding amino acids 8 to 37 of pp60^{src}, as previously described (17). Reagents were obtained as follows: protease inhibitors, paraformaldehyde, bovine serum albumin (fraction V), and detergents from Sigma Chemical Co.; fluorescein-conjugated goat anti-mouse immunoglobulin G, goat anti-rabbit immunoglobulin G, and normal goat serum from the Jackson Immunochemicals Co.; protein A conjugated to Sepharose CL-4B from Pharmacia Fine Chemicals; rhodamine-conjugated phalloidin from Molecular Probes Inc.; L-[^{35}S]methionine from ICN Radiochemicals; [^{3}H]myristic acid, 22.4 Ci/mmol (50 mCi/ml in dimethyl sulfoxide) from Du Pont, NEN Research Products; restriction enzymes, *Escherichia coli* DNA polymerase I, and T4 DNA ligase from New England BioLabs, Inc.; human serum fibronectin from Collaborative Research Inc.; and Gold Seal glass cover slips (18 by 18 mm; thickness, 1.5 mm) from VWR Scientific.

Metabolic labeling, immunoprecipitation, and subcellular fractionation. COS7 cells were labeled with L-[^{35}S]methionine for 18 h in Dulbecco modified Eagle medium containing 10% the normal concentration of L-methionine, 10% dialyzed fetal calf serum, and 200 μCi of L-[^{35}S]methionine per ml. COS7 cells were labeled with [^{3}H]myristic acid for 18 h in Dulbecco modified Eagle medium with 10% fetal calf serum, 1% dimethyl sulfoxide, and 500 μCi of [^{3}H]myristic acid per ml (5). Labeled cell extracts were prepared with [illegible] buffer (20 mM Tris hydrochloride [pH 8.0], 150 mM [illegible] rabbit sera and monoclonal antibodies were performed as described previously (21). Crude cell extracts prepared by Dounce homogenization of hypotonically swelled cells were fractionated into cytosol and membrane fractions by differential centrifuga-

* Corresponding author
[illegible]

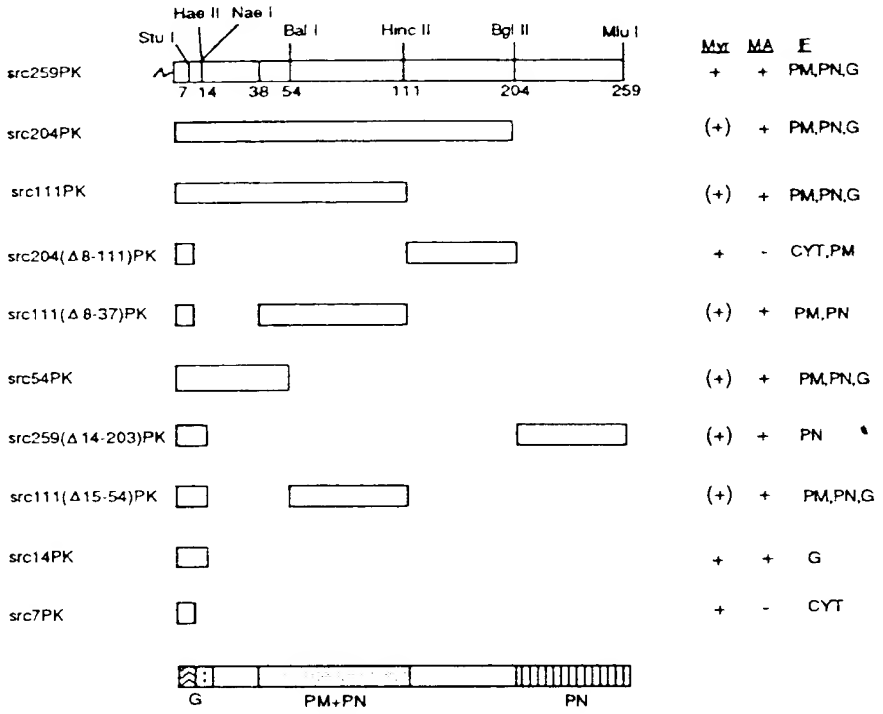


FIG. 1. Summary of the membrane association of *src*-PK proteins. The sequences derived from pp60^{src} in all of the *src*-PK proteins are depicted. The restriction sites used to create internal deletions of *src* sequences and to fuse the *src* sequences to the coding region of PK are indicated. Residue numbers correspond to the sequence of pp60^{src} encoded by the Prague C strain of Rous sarcoma virus (39). Myristylation (Myr) of hybrid proteins, when analyzed, is indicated by +. When *src*-PK proteins associated with membranes, myristylation was assumed [and denoted (+)] but not tested. Membrane association (MA) of hybrid proteins, as determined by biochemical fractionations, is indicated by +. The intracellular distribution of hybrid proteins was determined by immunofluorescence (IF). Association of hybrid proteins with plasma membranes (PM), with the cytoplasm (CYT), with perinuclear membranes (PN), or with cytoplasmic granules (G) is indicated. At the bottom of the figure the N-terminal 259 amino acids of pp60^{src} are divided into functional domains. The first 7 amino acids of pp60^{src} ( ) act as a recognition sequence for myristylation. Amino acids 1 to 14 cause association with cytoplasmic granules (G). Amino acids 38 to 111 ( ), together with the myristylation signal, cause association with both the plasma membrane and perinuclear membranes (PM+PN). Amino acids 204 to 259 ( ), together with the first 14 amino acids, causes association solely with perinuclear membranes (PN). The myristyl moiety is indicated by the crooked line in the diagram of *src*259PK (top of figure).

were quantitated by scanning autoradiograms with a Bio-Rad densitometer.

Construction of recombinant *src*-PK genes. The N-terminal amino acid sequence of the hybrid *src*-PK proteins is derived from pp60^{src} (B77 strain of Rous sarcoma virus), whereas the C-terminal amino acids are derived from PK. The *src*-PK proteins are designated by the C-terminal boundary of the domain of pp60^{src} that is fused to PK, and in cases in which this domain contains internal deletions, the amino acids that are lost are indicated in parentheses. Residue numbers reported correspond to the sequence of the Prague C strain of Rous sarcoma virus (40). The amino acids derived from the chicken M1 PK (residues 120 to 529) are the same in all cases (26). The nucleotide sequence encoding PK was derived

[illegible] acids of pp60^{src} were fused to the coding region of PK by cutting both genes with restriction endonucleases, repairing the ends with *E. coli* DNA polymerase I, and fusing the resulting blunt ends. Internal deletions of *src* sequences

sites used in these constructions are indicated in Fig. 1. The *Stu*I site at codon 7 is a restriction polymorphism which was created by converting lysine 7 to arginine (17). A simian virus 40 vector expressing the chicken fibroblast pp60^{src}, pSV5H, was constructed by replacing the PK cDNA of RL142PK10X with the c-*src* cDNA derived from p5H. Further details about the construction of these genes will be provided upon request.

Transfection of recombinant *src*-PK genes. COS7 cells were exposed for 18 h to calcium phosphate precipitates containing 20 μg of recombinant *src*-PK DNA and then subjected to a 2-min shock with Tris-buffered saline (0.8% NaCl, 0.1% glucose, 0.038% KCl, 0.2% Tris hydrochloride, 0.06% Tris base, 4.5 mg of phenol red per liter) containing 25% dimethyl

Immunofluorescence. [illegible] human serum fibronectin (10 μg/ml) in Dulbecco phosphate-buffered saline [PBS]) for 1 h at 37°C in a humidified chamber. Transfected COS7 cells were seeded onto glass cover slips 24 h after the transfection had begun. After 8 to

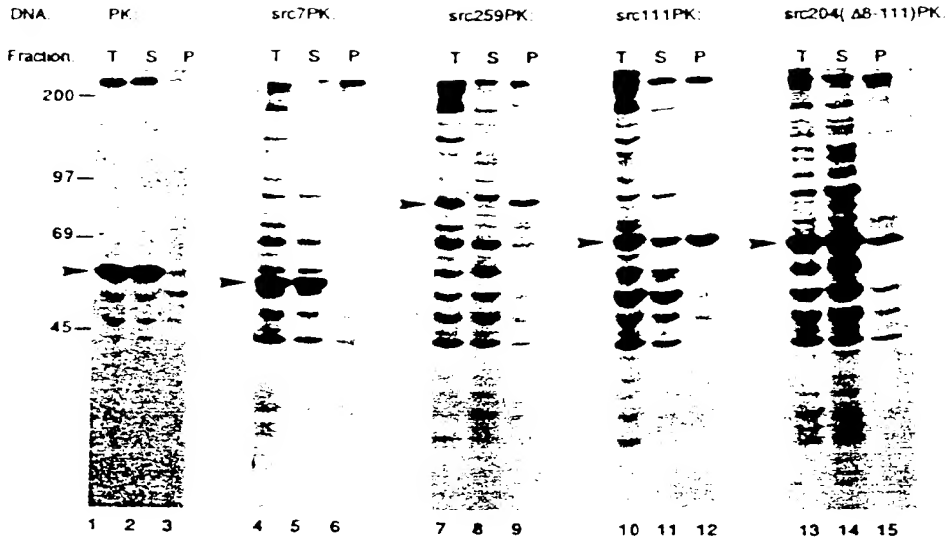


FIG. 2. The first 111 amino acids of pp60^{src} contain a membrane-anchoring domain. COS7 cells were transfected with plasmids encoding native PK (lanes 1 to 3), *src*7PK (lanes 4 to 6), *src*259PK (lanes 7 to 9), *src*111PK (lanes 10 to 12), or *src*204(Δ8–111)PK (lanes 13 to 15) as described in Materials and Methods. Transfected cells were labeled with L-[^{35}S]methionine for 12 h. Crude extracts (T; lanes 1, 4, 7, 10, and 13) were fractionated into cytosol (S; lanes 2, 5, 8, 11, and 14) and a membrane pellet (P; lanes 3, 6, 9, 12, and 15) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The positions of 200-, 97-, 69-, and 45-kDa molecular mass markers are indicated. The *src*-PK proteins are indicated by the arrowheads.

permeabilized with 0.1% Triton X-100 in PBS, and cover slips were coated with 10% normal goat serum in PBS to block nonspecific binding sites. Primary antibodies used were monoclonal anti-pp60^{src} 327 (10 μg/ml in PBS) and polyclonal rabbit anti-PK (1/200 dilution in PBS). Fluorescein-conjugated antibodies were used at a dilution of 1/200 in PBS. Rhodamine-conjugated phalloidin was used as recommended by the manufacturer. Stained cover slips were washed several times with PBS and then with absolute ethanol and were embedded in glycerol containing 2% propyl gallate, an antibleaching reagent. Microscopy was done with either a Zeiss Photomicroscope III or an inverted Olympus fluorescence microscope. Cells were photographed with Kodak Tri-X film at ASA 200. In general, 1 to 5% of the transfected COS7 cells gave a fluorescent signal following this procedure; this signal presumably corresponds to the fraction of cells that took up the DNA. The surrounding nonfluorescent cells acted as an internal control for the specificity of the antibodies used.

RESULTS

To test the role of N-terminal amino acid sequences of pp60^{src} in association with membranes and in subcellular localization, we constructed a set of genes encoding hybrid *src*-PK proteins (Fig. 1). Since myristylation is normally required for membrane [illegible]

Myristylation is not sufficient for membrane anchorage. We have previously shown that the first 7 amino acids of pp60^{src} act as a sufficient recognition sequence for myristylation [27]. Fusing sequences encoding the first 7 amino acids of [illegible]

*src*7PK, which encodes a myristylated protein (see Fig. 6). If myristylation is a sufficient cause of membrane association, the *src*7PK protein should attach to membranes.

Cells expressing either the native PK protein or the *src*7PK protein were fractionated into soluble and membrane fractions, and these fractions were assayed for PK proteins (Fig. 2). Virtually all of the native PK protein (Fig. 2, lanes 1 to 3) and the *src*7PK protein (Fig. 2, lanes 4 to 6) was recovered in the soluble fraction (Fig. 2, lane 2 and 5, respectively), indicating that a second domain of pp60^{src} is required in conjunction with myristylation to cause membrane association.

The gels illustrated in Fig. 2 contained variable amounts of proteins that appeared ancillary to the specific products of the transfected DNAs (marked by arrowheads). We do not know the identities of these ancillary proteins, but they were present in all specimens, including cells that had not been transfected. We therefore attribute them to nonspecific immunoprecipitation.

Anchoring PK to membranes with a domain from pp60^{src}. Since previous reports had implicated N-terminal sequences in pp60^{src} as membrane-anchoring domains (9, 12, 19, 22, 34), we fused sequences encoding the first 259 amino acids of the protein to the coding sequences of PK, creating a hybrid gene called *src*259PK. Cells expressing the *src*259PK protein [illegible]

[illegible] pp60^{src}, together with myristylation, are sufficient to mediate association with membranes.

The *src*259PK protein and pp60^{src} associate with membranes in a similar manner. Before further [illegible]

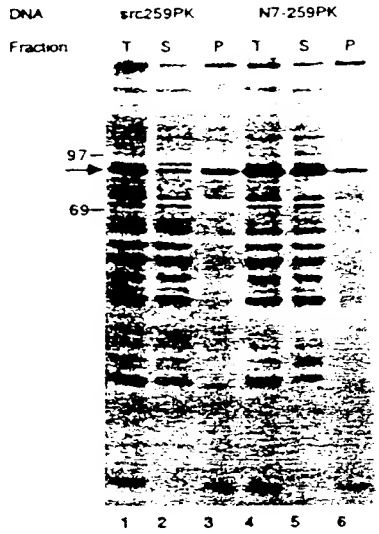


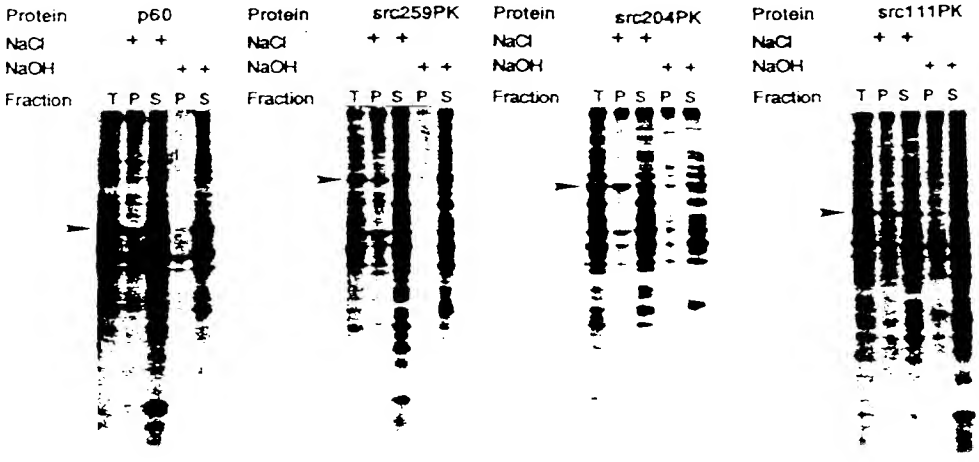
FIG. 3. Influence of myristylation of subcellular localization. COS7 cells were transfected with plasmids encoding either the *src*259PK protein (lanes 1 to 3) or the N7-259PK protein (lanes 4 to 6). Transfected cells were labeled with L-[^{35}S]methionine for 12 h. Crude extracts (T; lanes 1 and 4) were fractionated into cytosol (S; lanes 2 and 5) and a membrane pellet (P; lanes 3 and 6) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The *src*259PK protein is indicated by the arrow. The positions of 97- and 69-kDa molecular size markers are indicated.

259 amino acids of pp60^{src}, we tested whether the *src*259PK and pp60^{src} proteins associate with membranes in a similar manner. We first asked whether myristylation is required for the association of *src*259PK with membranes. Replacement of lysine 7 with an asparagine largely abolishes myristylation of pp60^{src} (17). Therefore, we converted lysine 7 to asparagine in *src*259PK and evaluated the subcellular distribution of the mutant protein (Fig. 3, lanes 4 to 6). The protein (designated N7-259PK) was found predominantly (ca. 87%) in the soluble fraction of cells (Fig. 3, lane 5), implying that the membrane association of *src*259PK, like that of pp60^{src} itself, requires N-terminal myristylation.

A second hallmark of the association of pp60^{src} with membranes is that, like other peripheral membrane proteins, pp60^{src} can be removed from membranes by alkaline extractions (Fig. 4, lanes 4 and 5). Similarly, the *src*259PK protein is extracted from membranes following extraction with base (Fig. 4, lanes 9 and 10). On the other hand, neither pp60^{src} (Fig. 4, lanes 2 and 3) nor *src*259PK (Fig. 4, lanes 6 to 8) is efficiently extracted from membranes by 0.3 M NaCl. We conclude that by several criteria, *src*259PK and pp60^{src} appear to associate with membranes in a similar manner.

The first 111 amino acids of the *src* protein are sufficient for membrane association. Previous work indicated that the N-terminal 10 kDa of pp60^{src} is required to anchor the protein to membranes (22, 34). To better define the responsible amino acid sequences, we fused the coding region of PK to sequences encoding each of the following domains: the first 204 amino acids of pp60^{src} (*src*204PK); the first 111 amino acids of pp60^{src} (*src*111PK); and the first 204 amino acids of pp60^{src}, from which residues 8 to 111 were deleted [*src*204(Δ8–111)PK]. Cells expressing these proteins were fractionated as described above (Fig. 2).

Although 75% of the *src*204(Δ8–111)PK protein was recovered in the cytosol (Fig. 2, lanes 13 to 15), most of the *src*204PK protein (not shown in Fig. 2, but see Fig. 4, lanes 11 to 15) and ca. 65% of the *src*111PK protein (Fig. 2, lanes 10 to 12) were recovered with the membrane pellet (Fig. 2, lanes 10 to 12). The proteins encoded by both *src*204PK (Fig. 4, lanes 11 to 15) and *src*111PK (Fig. 4, lanes 16 to 20) associated with membranes in a manner similar to pp60^{src},



[illegible] L-[^{35}S]methionine for 12 h. Crude extracts (T; lanes 1, 3, 11, and 16) were adjusted to either 0.3 M NaCl (lanes 2, 5, 7, 8, 12, 15, 17, and 18) or 0.3 M NaOH (lanes 4, 5, 9, 10, 14, 15, 19, and 20) and then fractionated into cytosol (S; lanes 3, 5, 8, 10, 13, 15, 18, and 20) and a membrane pellet (P; lanes 2, 4, 7, 9, 12, 14, 17, and 19) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with either MAb327 (protein A; lanes 2 to 5) or a rabbit polyclonal anti-PK antibody (lanes 6 to 20). The positions of

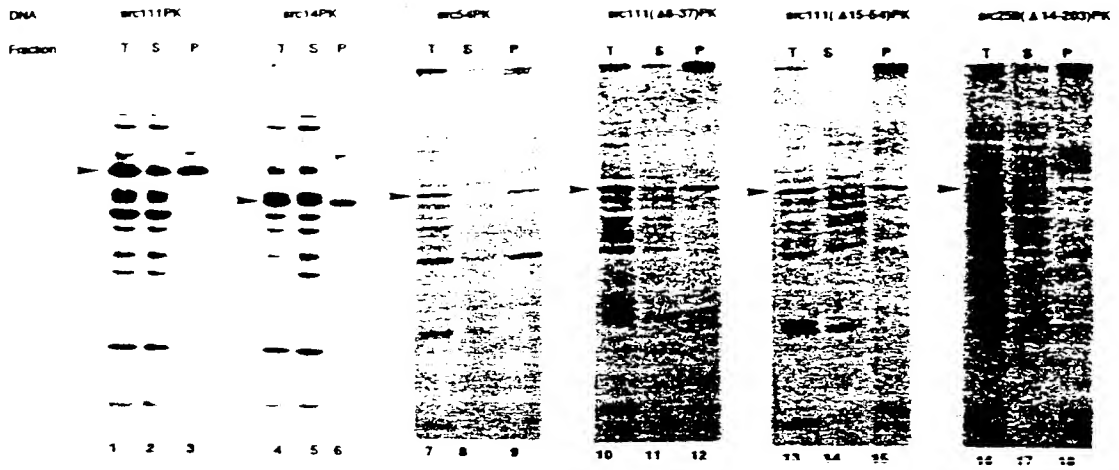


FIG. 5. The first 111 amino acids of pp60^{src} contain at least two membrane-anchoring domains. COS7 cells were transfected with plasmids encoding the *src*111PK (lanes 1 to 3), the *src*14PK (lanes 4 to 6), the *src*54PK (lanes 7 to 9), the *src*111(Δ8–37)PK (lanes 10 to 12), the *src*111(Δ15–54)PK (lanes 13 to 15), or the *src*259(Δ14–203)PK (lanes 16 to 18) proteins. Transfected cells were labeled with L-[^{35}S]methionine for 12 h. Crude extracts (T; lanes 1, 4, 7, 10, 13, and 16) were fractionated into cytosol (S; lanes 2, 5, 8, 11, 14, and 17) and a membrane pellet (P; lanes 3, 6, 9, 12, 15, and 18) by differential centrifugation. Equivalent amounts of each fraction were analyzed by immunoprecipitation with a polyclonal rabbit anti-PK antibody. The *src*-PK proteins are indicated by the arrowheads. Note that the *src*14PK protein migrates slightly farther than a background protein, which prevented quantitation of the membrane association of *src*14PK.

because they were extracted only partially from membranes by 0.3 M NaCl, but were extracted quantitatively by base.

We conclude that the first 111 amino acids of pp60^{src} are sufficient to cause association with membranes and that the association is similar to that found with native pp60^{src}. By contrast, amino acids 111 to 204, together with N-terminal myristylation, permit only limited association with membranes.

The first 111 amino acids of the *src* protein contain at least two independent membrane-anchoring domains. The domain represented by the first 111 amino acids of pp60^{src} was divided into several segments, and the sequences encoding these segments were fused to the coding region of PK. The association of these hybrid proteins with membranes was analyzed by biochemical fractionation as described above.

(i) The first 14 amino acids of the *src* protein contain a membrane-anchoring domain. It was previously shown that fusing the first 14 amino acids of pp60^{src} to any one of several other proteins causes these proteins to associate with membranes in crude biochemical fractionations (1, 31, 32). This result was confirmed by fusing sequences encoding the first 14 amino acids of pp60^{src} to the PK-encoding region, creating *src*14PK. An appreciable fraction of the *src*14PK protein was recovered with the membrane pellet after subcellular fractionation (Fig. 5, lanes 4 to 6), although precise quantitation was prohibited by the presence of a nearly comigrating protein.

Other hybrid proteins that contained the first 14 amino acids of pp60^{src} also associated with membranes: 72% of [illegible] to 54, 55 to 111, and 204 to 259 also contain membrane-anchoring domains, a possibility that will be confirmed below by immunofluorescence (see Fig. 7).

(ii) Amino acids 38 to 111 of the *src* protein contain a membrane-anchoring domain.

indicated that portions of pp60^{src} outside the first 14 amino acids contribute to membrane attachment. First, removal of more than 8 kDa from the amino terminus of pp60^{src} failed to detach the protein from membranes (22). Second, the protein encoded by the deletion mutant E3 *src*, which lacks amino acids 8 to 37, attaches to membranes (17).

We sought and identified a second membrane-anchoring domain by fusing the sequence encoding the first 81 amino acids of the E3 *src* protein to the coding region of PK, creating the *src*111(Δ8–37)PK gene. Most (66%) of the *src*111(Δ8–37)PK protein (Fig. 5, lanes 10 to 12) was recovered with the membrane pellet (Fig. 5, lane 12) following subcellular fractionation. This implies that amino acids 38 to 111 of pp60^{src} also contain a membrane-anchoring domain.

Failure of chimeric proteins to bind membranes cannot be attributed to poor myristylation. An alternative explanation for the behavior of the cytoplasmic *src*7PK protein and the membrane-associated *src*259PK and *src*14PK proteins is that they differ not by the presence of a membrane-anchoring domain, but rather in the stoichiometry of myristylation. If the association of pp60^{src} with membranes were mediated solely by the myristyl moiety, only the myristylated molecules would associate with membranes. This possibility was tested by comparing the stoichiometry of myristylation of the *src*7PK (Fig. 6, lanes 2 and 5), the *src*14PK (Fig. 6, lanes 3 and 6), and the *src*259PK (Fig. 6, lanes 1 and 4) proteins. Cells expressing these proteins were labeled with either L-[^{35}S]methionine (lanes 1 to 3) or [^{3}H]myristic acid (lanes 4

[illegible] value for the stoichiometry of myristylation of *src*259PK. In comparison, approximately 60% of the *src*14PK protein and 17% of the *src*7PK protein were myristylated (estimates derived from Fig. 6). Thus, if myristylation were sufficient to

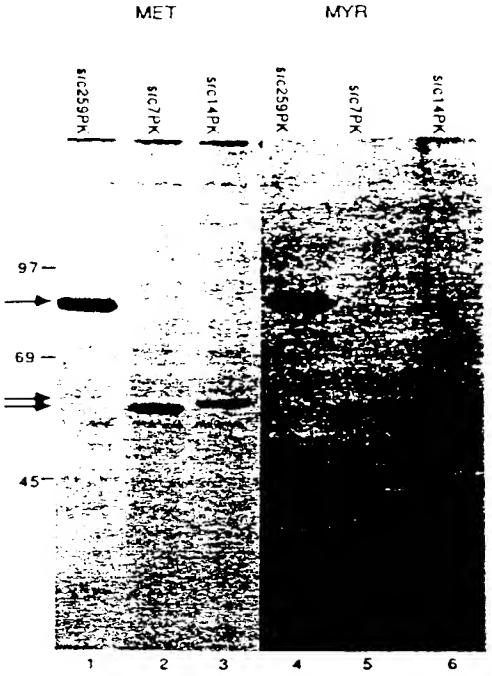


FIG. 6. The stoichiometries of myristylation of the *src*259PK, the *src*7PK, and the *src*14PK proteins are similar. COS7 cells were transfected with plasmids encoding the *src*259PK (lanes 1 and 4), the *src*7PK (lanes 2 and 5), or the *src*14PK (lanes 3 and 6) proteins. Transfected cells were labeled with either L-[^{35}S]methionine (lanes 1 to 3) or [^{3}H]myristic acid (lanes 4 to 6) for 12 h. Labeled cells were solubilized with lysis buffer, and PK proteins were immunoprecipitated with a polyclonal anti-PK antibody. The positions of 97-, 69-, and 45-kDa molecular mass markers are indicated. The PK proteins are indicated by the arrows. Stoichiometries mentioned in Results were calculated as follows, by using data from densitometry: $\frac{(\text{myr-x}/\text{myr-}src259)}{(^{35}\text{S-x}/^{35}\text{S-}src259)}$.

would be expected to be membrane associated, whereas only 3% was observed.

We also analyzed the membrane association of myristylated proteins. If the only relevant difference between the cytoplasmic *src*7PK and *src*204(Δ8–111)PK proteins, on the one hand, and the membrane-associated *src*259PK and *src*14PK proteins, on the other, were the proportion of the molecules that are myristylated, all of the myristylated molecules would be membrane associated. Cells expressing these proteins were labeled with [^{3}H]myristic acid and subsequently divided into membrane and cytosol fractions. Virtually all of the myristate-labeled *src*7PK protein was recovered in the cytoplasmic fraction, whereas most of the myristate-labeled *src*259PK protein and the *src*14PK protein

membranes does not reflect inefficient myristylation.

Localization of *src*-PK proteins by immunofluorescence. The intracellular distribution of pp60^{src} was previously analyzed by immunofluorescence and electron microscopy (30,

cells, pp60^{src} may be associated with a variety of subcellular compartments, including plasma membranes (typically at sites of focal adhesion), perinuclear membranes, and secretory granules. Therefore, we used immunofluorescence to characterize the intracellular distribution of *src*-PK proteins.

(i) pp60^{src} associates with plasma membranes, perinuclear membranes, and cytoplasmic granules in transfected COS7 cells. The distribution of pp60^{src} in COS7 cells following transfection with a plasmid containing the c-*src* gene was determined by immunofluorescence. The intracellular distribution of pp60^{src} in COS7 cells appeared to be a composite of three subcellular locations: plasma membranes, perinuclear membranes, and cytoplasmic granules (Fig. 7A).

(ii) The PK protein, the *src*7PK protein, and the *src*204(Δ8–111)PK protein are cytoplasmic. The intracellular distribution of the PK and hybrid proteins was analyzed by staining fixed cells with a polyclonal anti-PK antibody. The PK and *src*7PK proteins had a distribution representing the cytoplasm (Fig. 7B and C); the margins of cells expressing these proteins were poorly visualized after staining with anti-PK antibody, and the intensity of the staining was proportional to the thickness of the cell (i.e., to the volume of underlying cytoplasm). The *src*204(Δ8–111)PK protein appeared to be equally distributed between the plasma membrane and the cytoplasm (Fig. 7G). These results confirm our interpretation of the biochemical fractionations described above.

(iii) Most of the membrane-associated *src*-PK proteins have an intracellular distribution similar to pp60^{src}. The *src*259PK protein (Fig. 7D), the *src*204PK protein (Fig. 7E), the *src*111PK protein (Fig. 7F), the *src*111(Δ8–37)PK protein (Fig. 7H), the *src*54PK protein (Fig. 7I), and the *src*111(Δ15–54)PK protein (Fig. 7J) had a distribution similar to that of pp60^{src} itself. Often cells expressing *src*-PK proteins that associate with the plasma membrane appeared to have a filamentous component to the fluorescent signal (for example, see Fig. 7E). The filamentous structures also contained actin, as demonstrated by staining with rhodamine-conjugated phalloidin, an actin-specific stain (data not shown). We do not know the nature of these filamentous structures, but they conceivably could correspond to sites of adhesion.

(iv) The *src*259(Δ14–203)PK protein associates specifically with perinuclear membranes. Interestingly, the *src*259(Δ14–203)PK protein associated primarily with perinuclear membranes (Fig. 7K). Since the distribution of the *src*259(Δ14–203)PK and the *src*14PK (see below) proteins were different, we conclude that amino acids 204 to 259 contain a domain that preferentially targets proteins to perinuclear membranes.

(v) The *src*14PK protein associates with cytoplasmic granules. Cells expressing the *src*14PK protein had a punctate pattern of cytoplasmic fluorescence after staining with anti-PK antibody (Fig. 7L). These fluorescent spots had an apparent diameter of 0.2 μm and were uniformly distributed throughout the cytoplasm. We suspect that this distribution corresponds to a membranous organelle, rather than a large proteinaceous complex, because the *src*14PK protein does not sediment rapidly after dissolution of membranes with 1%

other cell lines support our conclusion that *src*14PK is associated principally in cytoplasmic granules, rather than in either of the other two locations reported here.)

The *src*14PK is the only hybrid protein that associates

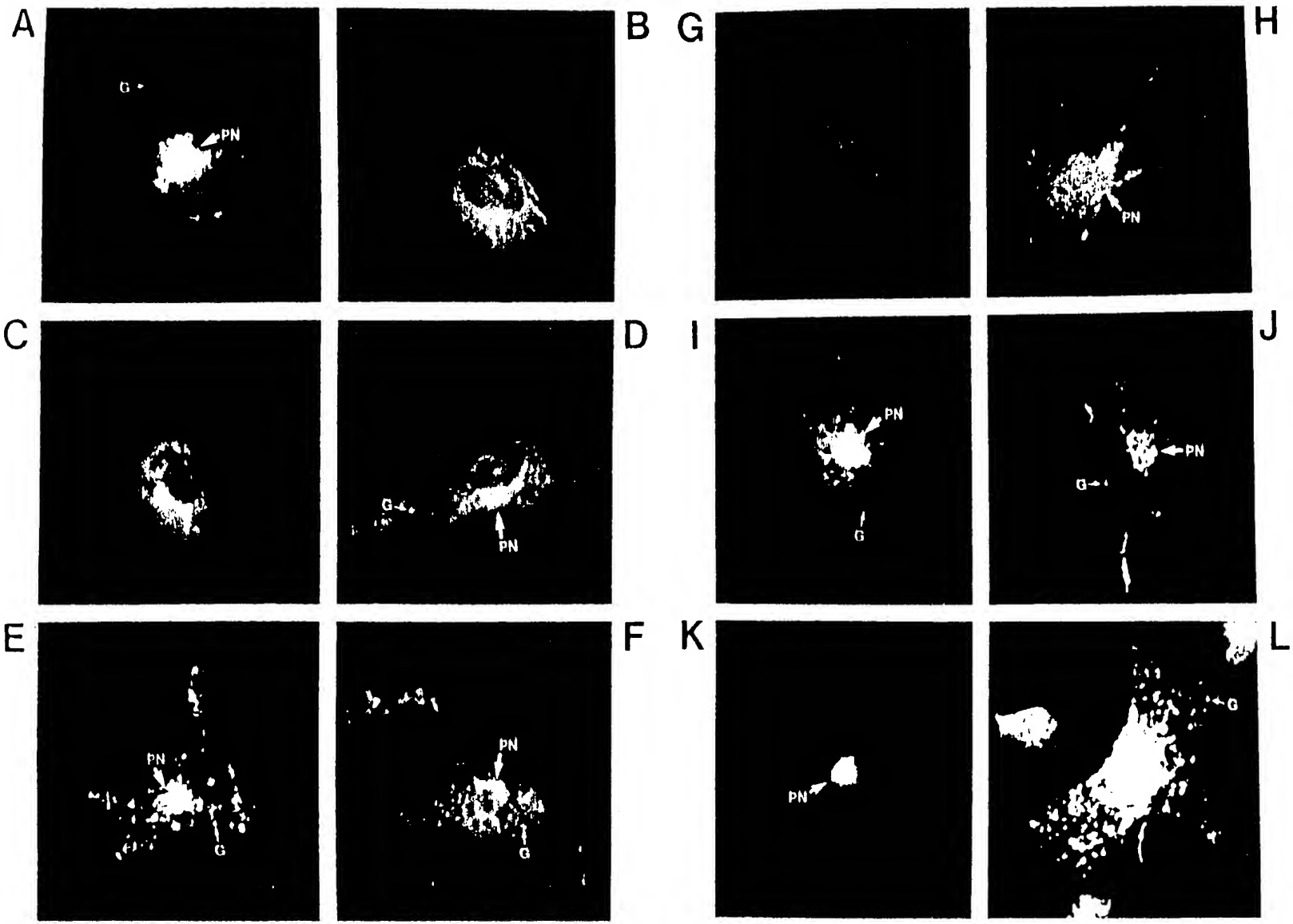


FIG. 7. Mem… nchoring domains target proteins to specific subcellular locations. COS7 cell were transfected with plasmids encoding pp60^{src} (A), PK (B), *src*7PK (C), …259PK (… (E), *src*111PK (F), *src*204(Δ8–111)PK (G), *src*111(Δ8–37)PK (H), *src*54PK (I), *src*111(Δ15–54)PK (J), *src*259(Δ14–203)PK (K), or *src*14PK (L) proteins. At 48 … after exp… DNAs, the cells were seeded onto glass cover slips. After an additional 24 h, the cells were fixed with paraformaldehyde, permeabilized with Triton X-100, …nd stained … nti-pp60^{src} MAb327 or a polyclonal anti-PK antibody, as described in Materials and Methods. Perinuclear membranes (PN) and cytoplasmic granules (G) are …dicated.

src-PK proteins that contain amino acids 1 to 14 were also found in cytoplasmic granules, proteins that contain additional portions of pp60^{src} associated with other membranes as well. For example, both the *src*54PK protein (Fig. 7I) and the *src*111(Δ15–54)PK protein (Fig. 7J) associated with the plasma membrane and perinuclear membranes, in addition to cytoplasmic granules. Because the distribution of these proteins differs from *src*14PK, amino acids 15 to 54 and 55 to 111 must contain additional sorting information. These results imply that multiple domains of pp60^{src} can affect the intracellular distribution of a protein.

DISCUSSION

Several lines of evidence suggest that the association of pp60^{src} with membranes is mediated in part by interaction between pp60^{src} and one or more membrane proteins. First, pp60^{src} is found in diverse subcellular locations that vary from one type of cell to another. These locations include the plasma membrane in fibroblasts (8, 21, 44), particularly focal contacts (37); perinuclear membranes in fibroblasts (36); cytoplasmic granules in platelets (33) and chromaffin cells (30); and growth comes in neurons (27, 41). These specificities suggest that pp60^{src} interacts with other proteins in the various locations. Second, reconstitution of purified pp60^{src} into lipid vesicles in vitro requires the addition of membrane proteins (34), and the binding of pp60^{src} to membranes utilizes at least one protein receptor that is both specific and saturable (35). Third, the protein tyrosine kinase encoded by *lck* associates with two transmembrane glycoproteins, the CD4 and CD8 antigens of T lymphocytes (38, 42), and the catalytic activity of *lck* can be modulated by cross-linking the CD4 antigen (43). Since the *lck* protein is closely related to pp60^{src}, it seems likely that the latter also interacts specifically with membrane proteins and that the interaction may help govern the activity of pp60^{src}.

If the attachment of pp60^{src} to membranes resembles that of the *lck* protein, the following predictions should be borne out: (i) pp60^{src} should behave like a peripheral membrane protein in schemes for solubilization; (ii) myristylation should not be sufficient for the association of pp60^{src} with membranes; (iii) specific domains of pp60^{src} should mediate its association with membranes; and (iv) these membrane-anchoring domains will target pp60^{src} to specific subcellular locations. The work reported here fulfills each of these predictions.

We acknowledge one caveat. Our work was performed with chimeric proteins formed between portions of pp60^{src} and the cytosolic protein PK. We cannot presently refute the possibility that the conformation (or other features) of these chimeras has influenced the outcome of our experiments in ways that are artifactual. It is reassuring, however, that the chimera *src*259PK (which contains all of the membrane-targeting domains that we have defined within pp60^{src}) distributes within cells in a manner identical to that of pp60^{src} itself. In addition, the conclusions reached here are [illegible]

[illegible] with membranes cannot be disrupted by chelating agents or by extreme concentrations of salt, whereas nonionic detergents completely extract pp60^{src} from membrane vesicles [21, 22]. These results led to the proposal and subsequent confirmation that pp60^{src} is a peripheral membrane protein, because it can be extracted from membrane vesicles with alkali. Similarly, hybrid *src*-PK proteins that are associated with membranes are also extracted with alkali. Perhaps extraction from membranes with alkali will be a general feature of myristylated proteins associated with membranes.

The role of myristylation in the membrane association of pp60^{src}. Myristylation is neither necessary nor sufficient for the association of pp60^{src} with membranes. Several forms of pp60^{src} are myristylated yet cytoplasmic (3, 11, 29). Conversely, nonmyristylated forms of pp60^{src} have been described which still associate with membranes (12, 20, 22). Our results provide further support for the notion that the myristyl moiety is not sufficient for the association of pp60^{src} with membranes: the myristylated proteins *src*7PK and *src*204(Δ8–111)PK are cytoplasmic when analyzed by either biochemical fractionation or immunofluorescence (see Fig. 1 for a summary).

What, then, is the role of myristylation in the attachment of pp60^{src} to membranes? First, the modification may increase the affinity of the protein for membranes: forms of pp60^{src} that lack myristylation but are nevertheless found on membranes can be solubilized by ionic strength alone. Second, myristylation may initiate the binding of pp60^{src} to membranes: typically, nonmyristylated forms of pp60^{src} never attach to membranes. The full association of pp60^{src} with membranes requires one or more portions of the protein itself, however, and once the association is established, it may be independent of myristylation: cleavage of membrane-bound pp60^{src} with protease removes 8 kDa from the amino terminus, yet fails to disrupt the association of the remainder of the protein with the membranes (22).

Membrane-anchoring domains target molecules to distinct subcellular locations. Previous reports have demonstrated that pp60^{src} is found in plasma membranes, perinuclear membranes, and cytoplasmic granules. The results reported here are unusual in that pp60^{src} is found in all of these locations in a single type of cell, a circumstance that we cannot presently explain but that might be due either to the use of COS7 cells or to the exceptional abundance of pp60^{src} produced in the cells (10- to 20-fold more than found in cells transformed by Rous sarcoma virus [data not shown]). Whatever its cause, this circumstance allowed us to demonstrate that distinctive domains of pp60^{src} are required for targeting the protein to each subcellular location.

Amino acids 1 to 14 of pp60^{src} cause proteins to associate primarily with cytoplasmic granules in COS7 cells. Perhaps these amino acids specifically target pp60^{src} to granules in chromaffin cells and platelets as well. Amino acids 38 to 111 cause proteins to associate with both plasma membranes and perinuclear membranes. We suspect that these amino acids make up multiple membrane-anchoring domains, because in conjunction with amino acids 1 to 14, either amino acids 15 to 54 or amino acids 55 to 111 are sufficient to cause association with plasma membranes, perinuclear membranes, and cytoplasmic granules. Amino acids 204 to 259, together with amino acids 1 to 14, cause a protein to

[illegible] the subcellular distribution of pp60^{src} controlled by **phosphorylation?** How is it that in most types of cells pp60^{src} is localized to one subcellular compartment and not to others? It appears possible that the activity of membrane-anchoring domains is regulated by phosphorylation. For

target proteins specifically to the membranes of cytoplasmic granules. Within and close to this domain are serine residues that are known to be phosphorylated by cellular kinases: serine 12 by kinase C (13, 14) and serine 17 by the cyclic AMP-dependent protein kinase (6, 10, 18). Perhaps phosphorylation of these sites helps regulate the subcellular location of $pp60^{src}$. There is precedent for this speculation in the finding that phosphorylation of the epidermal growth factor receptor by kinase C causes internalization of receptor molecules (24).

The viral and cellular *src* proteins differ in their amino acid sequence, including regions used here in chimeras. Since the chimeras contained sequences derived only from viral *src*, the conclusions that we have reached regarding the targeting of *src* protein to membranous compartments apply rigorously only to the viral version of the protein. Alternative splicing in neurons results in the synthesis of a novel form of $pp60^{src}$ ($pp60^{+}$) (23, 28). Perhaps the localization of $pp60^{+}$ in neurons to growth cones is in part due to these structural alterations.

Where within the cell is the transforming activity of $pp60^{src}$ effected? Transformation by the viral version of $pp60^{src}$ requires that the protein be associated with membranes (see reference 19 for a review), presumably because the crucial substrates of $pp60^{src}$ are also membrane associated. Since $pp60^{src}$ is capable of associating with various membranes, it is not clear whether transformation results from the activity of $pp60^{src}$ at a single or at multiple subcellular locations.

Surprisingly, many of the mutant *src* proteins with deletions that begin at codon 15 retain transforming activity. None of these proteins has been analyzed by immunofluorescence. Our results indicate that these transforming *src* proteins would associate primarily with perinuclear membranes and cytoplasmic granules.

No mutant allele of *src* has been described in which all of the membrane-anchoring domains have been deleted. In most of the available mutants, the encoded proteins retain the first 14 amino acids of $pp60^{src}$; among these mutant proteins, only the product of the NY18-3 allele is not associated with membranes (11). The NY18-3 *src* gene lacks only the sequences that normally encode amino acids 169 to 264, yet it encodes a myristylated protein that is located in the cytoplasm. Our results do not explain these properties. Perhaps the conformation of this protein precludes association with membranes.

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is attached to the transforming protein of Rous sarcoma virus during or immediately after synthesis and is present in both soluble and membrane-bound forms of the protein. Mol. Cell. Biol. 4:2697–2704.

4. **Buss, J. E., and B. M. Sefton.** 1984. Myristic acid, a rare fatty acid, is the lipid attached to the transforming protein of Rous sarcoma virus and its cellular homolog. J. Virol. 53:7–12.
5. **Chow, M., J. E. Newman, D. Filman, J. M. Hogle, D. J. Rowlans, and F. Brown.** 1987. Myristylation of picornavirus capsid protein VP4 and its structural significance. Nature (London) 327:482–486.
6. **Collett, M. S., E. Erikson, and R. L. Erikson.** 1979. Structural analysis of the avian sarcoma virus transforming protein: sites of phophorylation. J. Virol. 29:770–781.
7. **Courtneidge, S. A., and J. M. Bishop.** 1982. Transit of $pp60^{src}$ to the plasma membrane. Proc. Natl. Acad. Sci. USA 79:7117–7121.
8. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{proto\text{-}src}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. USA 77:3783–3787.
9. **Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa.** 1984. A short sequence in the $p60^{src}$ N terminus is required for $p60^{src}$ myristylation and membrane association and for cell transformation. Mol. Cell. Biol. 4:1834–1842.
10. **Cross, F. R., and H. Hanafusa.** 1983. Local mutagenesis of Rous sarcoma virus: the major sites of tyrosine and serine phosphorylation for $pp60^{src}$ are dispensable for transformation. Cell 34:597–607.
11. **Garber, E. A., F. R. Cross, and H. Hanafusa.** 1985. Processing of $p60^{v\text{-}src}$ to its myristylated membrane-bound form. Mol. Cell. Biol. 5:2781–2788.
12. **Garber, E. A., and H. Hanafusa.** 1987. NH_2-terminal sequences of two *src* proteins that cause aberrant transformation. Proc. Natl. Acad. Sci. USA 84:80–85.
13. **Gentry, L. E., K. E. Chaffin, M. Shoyab, and A. F. Purchio.** 1986. Novel serine phosphorylation of $pp60^{v\text{-}src}$ in intact cells after tumor promoter treatment. Mol. Cell. Biol. 6:735–738.
14. **Gould, K. L., J. R. Woodgett, J. A. Cooper, J. E. Buss, D. Shalloway, and T. Hunter.** 1985. Protein kinase C phosphorylates $pp60^{src}$ at a novel site. Cell 42:849–857.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467.
16. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino sequence able to specify nuclear location. Cell 39:499–509.
17. **Kaplan, J. M., G. Mardon, J. M. Bishop, and H. E. Varmus.** 1988. The first seven amino acids encoded by the v-*src* oncogene act as a myristylation signal: lysine-7 is a critical determinant. Mol. Cell. Biol. 8:2435–2441.
18. **Karess, R. E., and H. Hanafusa.** 1981. Viral and cellular *src* genes contribute to the structure of recovered avian sarcoma virus transforming protein. Cell 24:155–164.
19. **Krueger, J. G., E. A. Garber, and A. R. Goldberg.** 1983. Subcellular localization of $pp60^{src}$ in RSV transformed cells. Curr. Top. Microbiol. Immunol. 107:51–124.
20. **Krueger, J. G., E. A. Garber, A. R. Goldberg, and H. Hanafusa.** 1982. Changes in amino-terminal sequences of $pp60^{src}$ lead to decreased membrane association and decreased *in vivo* tumorigenicity. Cell 28:889–896.
21. Krueger, J. G., E. Wang, and A. R. Goldberg. 1980. Evidence

2. **Buss, J. E., M. P. Kamps, K. Gould, and B. M. Sefton.** 1986. The absence of myristic acid decreases membrane binding of $p60^{src}$ but does not affect tyrosine protein kinase activity. J. Virol. 232:468–474.

78:3624–3628.

28. Levy, J. B., T. Dorai, L.-H. Wang, and J. S. Brugge. 1987. The structurally distinct form of $pp60^{src}$ detected in neuronal cells is encoded by a unique *src* mRNA. Mol. Cell. Biol.

24. **Lin, C. R., W. S. Chen, C. S. Lazar, C. D. Carpenter, G. N. Gill, R. M. Evans, and M. G. Rosenfeld.** 1986. Protein kinase C phosphorylation at Thr 654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms. Cell **44**:839–845.
25. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48**:352–360.
26. **Lonberg, N., and W. Gilbert.** 1983. Primary structure of chicken pyruvate kinase mRNA. Proc. Natl. Acad. Sci. USA **80**:3661–3665.
27. **Maness, P. F., M. Aubry, C. G. Shores, L. Frame, and K. H. Pfenninger.** 1988. c-*src* gene product in developing rat brain is enriched in nerve growth cone membranes. Proc. Natl. Acad. Sci. USA **85**:5001–5006.
28. **Martinez, R., B. Mathey-Prevot, A. Bernards, and D. Baltimore.** 1987. Neuronal $pp60^{c\text{-}src}$ contains a six-amino acid insertion relative to its non-neuronal counterpart. Science **237**:411–415.
29. **Olson, E. N., D. A. Towler, and L. Glaser.** 1985. Specificity of fatty acid acylation of cellular proteins. J. Biol. Chem. **260**: 3784–3790.
30. **Parsons, S. J., and C. E. Creutz.** 1986. $p60^{c\text{-}src}$ activity detected in the chromaffin granule membrane. Biochem. Biophys. Res. Commun. **134**:736–743.
31. **Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa.** 1985. Fine structural mapping of a critical NH_2-terminal region of $p60^{src}$. Proc. Natl. Acad. Sci. USA **82**:1623–1628.
32. **Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa.** 1985. An N-terminal peptide from $p60^{src}$ can direct myristylation and plasma membrane localization when fused to heterologous proteins. Nature (London) **314**:374–377.
33. **Rendu, F., M. Lebret, S. Danielian, R. Fagard, S. Levy-Toledano, and S. Fischer.** 1989. High pp60-src level in human platelet dense bodies. Blood **73**:1545–1551.
34. **Resh, M. D.** 1988. Reconstitution of the Rous sarcoma virus transforming protein $pp60^{v\text{-}src}$ into phospholipid vesicles. Mol. Cell. Biol. **8**:1896–1905.
35. **Resh, M. D.** 1989. Specific and saturable binding of $pp60^{v\text{-}src}$ to plasma membranes: evidence for a myristyl-*src* receptor. Cell **58**:281–286.
36. **Resh, M. D., and R. L. Erikson.** 1985. Highly specific antibody to Rous sarcoma virus *src* gene product recognizes a novel population of $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ molecules. J. Cell Biol. **100**:409–418.
37. **Rohrschneider, L. R.** 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the *src* gene product. Proc. Natl. Acad. Sci. USA **77**:3514–3518.
38. **Rudd, C. E., J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman.** 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. USA **85**:5190–5194.
39. **Schultz, A. M., L. E. Henderson, S. Oroszlan, E. A. Garber, and H. Hanafusa.** 1985. Amino terminal myristylation of the protein kinase p60*src*, a retroviral transforming protein. Science **227**: 427–429.
40. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–869.
41. **Sobue, K., and K. Kanda.** 1988. Localization of $pp60^{c\text{-}src}$ in growth cone of PC12 cells. Biochem. Biophys. Res. Commun. **157**:1383–1390.
42. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell **55**:301–308.
43. **Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen.** 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. Nature (London) **338**:257–259.
44. **Willingham, M. C., G. Jay, and I. Pastan.** 1979. Localization of the ASV *src* gene product to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell **18**:125–134.

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Myristic Acid Is Attached to the Transforming Protein of Rous Sarcoma Virus During or Immediately After Synthesis and Is Present in Both Soluble and Membrane-Bound Forms of the Protein

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Myristic acid, a minor component of cellular fatty acids, has been shown previously to be covalently bound to most molecules of $p60^{src}$, the transforming protein of Rous sarcoma virus. We have now determined at what time during the life cycle of $p60^{src}$, and where within the cell, this lipid becomes attached to the protein. $p60^{src}$ was found to acquire myristic acid at only one time, during or immediately after its synthesis. $p60^{src}$ is known to be synthesized on free polysomes and appears at the cytoplasmic face of the plasma membrane after a lag of 10 min. The addition of myristic acid to $p60^{src}$ therefore precedes the binding of the protein to the plasma membrane. The lipid attached to $p60^{src}$ is a permanent, metabolically stable part of the protein; we found no evidence for turnover of the myristyl moiety. However, we did find myristate attached to various soluble forms of $p60^{src}$ and to a large number of cytosolic cellular proteins as well. This demonstrates that the attachment of myristic acid to a protein is not in itself sufficient to convert a soluble protein into a membrane-bound protein.

$p60^{src}$, the transforming protein of Rous sarcoma virus (RSV) (5) is a protein kinase which phosphorylates tyrosine residues in substrate proteins (7, 16, 22). $p60^{src}$ undergoes an unusual form of protein modification: the 14-carbon saturated fatty acid, myristic acid, is covalently attached to its amino terminus (39; J. E. Buss and B. M. Sefton, J. Virol., in press). In these studies we have characterized the time and intracellular location of myristic acid addition to $p60^{src}$ and analyzed the effect of temperature-sensitive mutations on this process.

Although few studies have examined the biochemistry of fatty acylation of proteins, two different types of modification are known to occur. The 16-carbon saturated fatty acid, palmitic acid, has been shown to be present in a variety of membrane-associated proteins. These include the transferrin receptor (24); $p21^{ras}$, the transforming protein of Harvey murine sarcoma virus (39); and the E1 and E2 glycoproteins of Sindbis virus and the G glycoprotein of vesicular stomatitis virus (31, 32). The palmityl group is attached to these proteins via ester bonds to cysteine (30) or possibly serine residues (32). The addition of ester-linked palmitate is clearly a posttranslational event. The fatty acid is added to viral glycoproteins in the Golgi apparatus ca. 20 min after their synthesis (33), and only the mature forms of $p21^{ras}$ and the transferrin receptor contain palmitic acid (25, 39).

Only five identified proteins are known to contain a covalently attached myristic acid. These are the cellular and viral forms of $p60^{src}$ (Buss and Sefton, in press), the catalytic subunit of the cyclic AMP-dependent protein kinase (6), the protein phosphatase, calcineurin B (1), the T-cell-specific p56 protein from LSTRA cells (41), and proteins which contain the $p15^{gag}$ protein of mammalian retroviruses (15, 35). This last group includes the unglycosylated forms of a

residue. It is not known at what time during the life span of

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the polypeptide or where within the cell myristic acid becomes attached to these proteins.

Shortly after synthesis on free polyribosomes (21, 29), $p60^{src}$ forms a complex with two cellular proteins (3, 4, 8): a 90,000-dalton phosphoprotein (hsp90) whose synthesis is increased by stress (26) and a 50,000-dalton phosphoprotein (p50) which contains both phosphoserine and phosphotyrosine (2, 3, 14, 16, 27). Courtneidge and Bishop (8) and Brugge et al. (4) have suggested that this complex may act as a system to transport newly synthesized $p60^{src}$ to the plasma membrane where the majority of the $p60^{src}$ molecules reside (9, 19, 20). We have examined how soon after synthesis the myristyl group is added to $p60^{src}$ in two ways. First, we have determined whether the $p60^{src}$ found in the complex with hsp90 and p50 contains myristic acid. Second, we have measured the effect of the inhibition of protein synthesis on the incorporation of [^{3}H]myristic acid into the protein. We also have investigated the half-life of the myristyl moiety of $p60^{src}$ to determine whether myristylation is a reversible phenomenon.

The amount of lipid in the temperature-sensitive $p60^{src}$ protein of *ts*NY68 has been reported to be decreased significantly at the nonpermissive temperature (13). These studies, however, used [^{3}H]palmitic acid to quantify the amount of fatty acid in $p60^{src}$. Since all of the ^{3}H incorporation into $p60^{src}$ from [^{3}H]palmitic acid appears to result from catabolism of the palmitate to myristic acid (Buss and Sefton, in press), we have reexamined the effect of temperature-sensitive mutations on the addition of lipid to $p60^{src}$ with [^{3}H]myristic acid as the biosynthetic precursor.

We initially suggested that the lipid bound to $p60^{src}$ might help anchor the protein to the cytoplasmic face of the plasma membrane (39). This idea was supported by the report that

cellular proteins. We find that many myristylated proteins, including a subpopulation of $p60^{src}$, behave as soluble proteins during traditional cell fractionation. Attachment of

myristic acid to a protein thus does not induce an irreversible association of the protein with cell membranes.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo cell cultures were prepared and infected with Schmidt-Ruppin RSV, subgroup D (SR-RSV-D); with SR-RSV, subgroup A (SR-RSV-A); with a mutant virus derived from SR-RSV-A, *ts*NY68 (17), which is temperature sensitive for transformation; or with a temperature-sensitive mutant of Prague RSV-A, *ts*LA29 (42), as described previously (37). Cells infected with SR-RSV-D were maintained at 41°C and subcultured the day before use in the Dulbecco-Vogt modification of Eagle medium supplemented with 2% tryptose phosphate broth and 4% calf serum. Cells infected with SR-RSV-A, *ts*NY68, or *ts*LA29 were maintained at 37°C for 3 days, at which time ca. 50% of the wild-type RSV-infected cells were morphologically transformed. Infected cells were then subcultured and allowed to grow for 2 more days at either 35 or 41°C in the same medium as the cells infected with SR-RSV-D.

Labeling with [^{3}H]palmitic acid, [^{3}H]myristic acid, and [^{35}S]methionine. 9,10-[^{3}H]palmitic acid (23.5 Ci/mmol; New England Nuclear Corp.) in toluene or 9,10-[^{3}H]myristic acid (12.9 Ci/mmol, New England Nuclear Corp.) in ethanol was dried and dissolved in dimethyl sulfoxide as described previously (39). The complete medium for labeling with fatty acids consisted of the Dulbecco-Vogt modified Eagle medium, 10% calf serum, 5 mM sodium pyruvate, nonessential amino acids, 1% dimethyl sulfoxide, and the indicated amounts of fatty acid. Cells were labeled with 0.1 mCi of [^{35}S]methionine per ml in methionine-free Dulbecco-Vogt modified Eagle medium containing 10% dialyzed calf serum. Labeling was for 2 h at 41°C in the case of SR-RSV-D-infected cells or for 2 h at 35 or 41°C as indicated in the case of cells infected with SR-RSV-A or mutant viruses.

Treatment with cycloheximide. Cells were treated with cycloheximide by replacement of their growth medium with fresh medium containing 20 μg of cycloheximide per ml. Control cultures also received fresh medium. The treated cells were then labeled for 2 h in radioactive medium which contained cycloheximide.

Immunoprecipitation and SDS-PAGE. Lysis of cells in RIPA buffer and immunoprecipitation with rabbit anti-RSV tumor sera were as described previously (36). For analysis of total cell protein, cultures labeled with [^{3}H]myristic acid or [^{3}H]palmitic acid were dissolved by adding electrophoresis sample buffer directly to a washed cell monolayer. Samples were transferred to tubes, boiled for 1 min, and then passed through a 27-gauge needle to shear cellular DNA. All samples were analyzed by electrophoresis on 15% polyacrylamide gels (37).

Sedimentation analysis of p60^{src}. For glycerol gradient analysis, 10^6 cells were lysed in 200 μl of Nonidet P-40 (NP-40) buffer (37) (1% NP-40, 0.15 M NaCl, 10 mM sodium phosphate [pH 7.2], 2 mM EDTA, 1% Trasylol). The lysate was clarified by centrifugation at 20,000 × *g* for 30 min and layered onto a 2.4-ml gradient of 5 to 20% glycerol in NP-40 buffer formed on top of 0.25 ml of 50% glycerol in NP-40 buffer. Centrifugation was for 19 h at 40,000 rpm (150,000 × *g*) at 4°C in a Beckman SW50 [illegible]. Gradients were collected from the bottom of the tube by puncture [illegible] of the precipitation [illegible]

containing 5 mM KCl, 1 mM $MgCl_2$, and 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.0) and broken with 30 strokes in a tight-fitting Dounce homogenizer (9). The lysate was adjusted to 0.1 M NaCl (19), and soluble and particulate fractions were prepared directly from the cell lysate by centrifugation in Beckman Ultraclear tubes at 100,000 × *g* for 30 min at 4°C.

Fluorography and quantification of radioactive proteins. Polyacrylamide gels were impregnated with diphenyloxazole, dried, and exposed to presensitized X-ray film at −70°C. The relative amount of p60^{src} in separate lanes of the gel was quantified by scanning films with a Quick-Scan densitometer (Helena Laboratories) and calculating peak areas from the integrator tracings. For proteins labeled with [^{3}H]palmitic acid in the presence or absence of cycloheximide, scans of entire gel channels were cut from the chart paper and weighed. [^{3}H]myristic acid and [^{35}S]methionine incorporation into p60^{src} from *ts*NY68 cells was determined by excising pieces of the dried, diphenyloxazole-impregnated polyacrylamide gel and counting them directly in 3a70B scintillation fluid (Research Products International Corp.).

RESULTS

p60^{src} in the complex with hsp90 and p50 cellular proteins contains myristic acid. Chicken cells transformed with SR-RSV-A and grown at 41°C were labeled for 2 h with either [^{3}H]myristic acid or [^{35}S]methionine and fractionated by glycerol gradient sedimentation. p60^{src} was recovered from each gradient fraction by immunoprecipitation (Fig. 1). A total of 65% of the [^{35}S]methionine-labeled p60^{src} sedimented as a monomer (fractions 12 to 14); 35% was present in a more rapidly sedimenting complex with hsp90 and p50 (fractions 7 to 8). [^{3}H]myristic acid-labeled p60^{src} isolated by immunoprecipitation was distributed in exactly the same manner: 69% as a monomer and 31% in the complex. Neither hsp90 nor p50 contained myristate. Immunoprecipitation with this rabbit anti-RSV tumor serum detects ~25% of the p60^{src} present in a cell lysate (Buss and Sefton, in press). So few proteins contain myristic acid that p60^{src} also can be identified directly in cell lysates without immunoprecipitation (Buss and Sefton, in press). To ensure that we were not looking at a restricted subpopulation of p60^{src}, we also examined the sedimentation properties of the total population of [^{3}H]myristic acid-labeled p60^{src} directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A total of 61% of the [^{3}H]myristic acid-labeled p60^{src} sedimented as a monomer, and 39% was found in the high-molecular-weight complex. Both procedures thus show that the soluble, complex-bound form of p60^{src} contains myristic acid and suggest that myristylation of p60^{src} occurs during or before formation of the complex.

Addition of myristic acid to p60^{src} occurs rapidly and requires continuing protein synthesis. Because newly synthesized p60^{src} is found exclusively in the soluble complex with hsp90 and p50 and only later associates with cellular membranes, the presence of a myristyl group attached to p60^{src} in the complex raised the possibility that myristic acid was added to the protein before its initial interaction with membranes. The time of addition of myristic acid to p60^{src} was therefore examined by a second method. We inhibited the

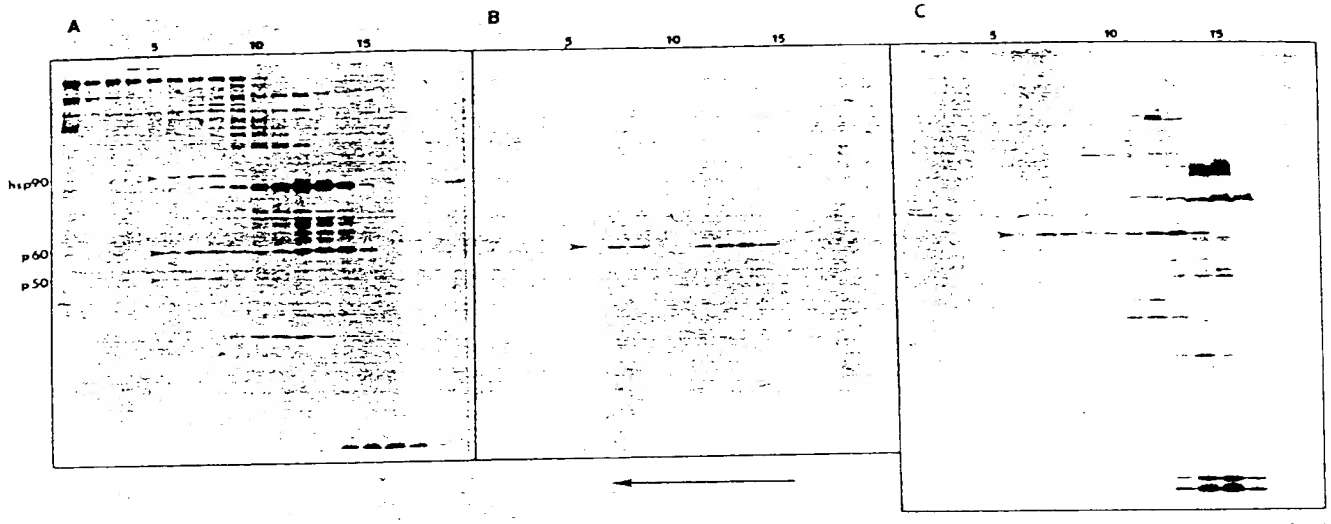

FIG. 1. [^{3}H]myristic acid-labeled p60^{src} is present in the complex with hsp90 and p50. SR-RSV-A-infected chicken cells were labeled for 2 h with [^{35}S]methionine or [^{3}H]myristic acid. Cells were lysed in NP-40 buffer, and the lysates were sedimented at 150,000 × *g* for 19 h at 4°C in gradients of 5 to 20% glycerol in NP-40 buffer. Proteins immunoprecipitated from gradient fractions with antitumor serum were analyzed by SDS-PAGE and visualized by fluorography. Gradient fractions from the [^{3}H]myristic acid-labeled cells were also analyzed by SDS-PAGE without immunoprecipitation. Exposure time was 6 h for [^{35}S]methionine-labeled proteins, 4 days for [^{3}H]myristic acid-labeled immunoprecipitated proteins, and 12 days for total [^{3}H]myristic acid-labeled proteins. hsp90 and p50 are indicated with arrows. The direction of sedimentation was from right to left and is indicated at the bottom of the figure. Fraction numbers are indicated at the top of the fluorograms. A, Immunoprecipitated proteins from [^{35}S]methionine-labeled cells; B, immunoprecipitated proteins from [^{3}H]myristic acid-labeled cells; C, total [^{3}H]myristic acid-labeled proteins.

untreated cells (Fig. 2). The lack of protein myristylation in the presence of cycloheximide was not due to inhibition of uptake or activation of myristic acid, for the amount of [^{3}H]myristic acid incorporated into phosphatidylcholine was the same for treated and control cultures (data not shown). To measure more precisely how soon myristylation occurs after the release of p60^{src} from the polysome, we decreased the time of pretreatment of the cells with cycloheximide. Even when cycloheximide and [^{3}H]myristic acid were added to the cells simultaneously, the incorporation of [^{3}H]myristic acid into p60^{src} and all but three other cellular proteins was abolished (Fig. 2). An immediate cessation of myristate incorporation into protein also occurred in cells treated with emetine (data not shown). p60^{src} molecules synthesized before the addition of the cycloheximide did not incorporate [^{3}H]myristic acid. Myristylation thus appears to occur only during or immediately after synthesis of the polypeptide for p60^{src} and the large majority of cellular myristic acid-containing proteins.

In contrast to the nearly total inhibition of protein myristylation, the attachment of palmitic acid to cellular proteins was less affected by cycloheximide (Fig. 2). Densitometric analysis indicated that exposure to cycloheximide for 20 min decreased the total incorporation of [^{3}H]palmitic acid into the cellular proteins resolved on our gels by 75%. The labeling of at least three minor proteins with molecular weights between 30,000 and 46,000 was unaffected by cycloheximide. When [^{3}H]palmitic acid was added simultaneously

not replaced. To examine the stability of the myristyl group of p60^{src} in another way, we measured the half-life of [^{3}H]myristic acid-labeled p60^{src}. Cells were labeled with [^{3}H]myristic acid or [^{35}S]methionine for 2 h and then incubated in nonradioactive medium for 3, 6, 10, or 22 h (Fig. 3). The amount of [^{35}S]methionine-labeled p60^{src} decreased by 24% in 3 h and by 98% in 22 h. The polypeptide chain of p60^{src} was therefore degraded with a half-life of ca. 7 h, in agreement with previous results (38). The amount of [^{3}H]myristic acid-labeled p60^{src} decreased by 28% in 3 h and by more than 95% in 22 h. The half-life of the myristate in p60^{src} thus appeared to be the same as the half-life of the protein itself. Because it is difficult to dilute the intracellular pool of radioactive lipids rapidly, continued incorporation of [^{3}H]myristic acid during the chase could potentially mask the loss of the myristyl moiety. Because the amount of [^{3}H]myristic acid in p60^{src} did, however, decrease substantially within the first 3 h, the extent of continued incorporation of [^{3}H]myristic acid after initiation of the chase would appear to be small. The myristyl group is therefore as metabolically stable as the polypeptide backbone of the p60^{src} protein.

Soluble cytoplasmic proteins can contain myristic acid. Both newly synthesized p60^{src} and the population of p60^{src} in the complex with hsp90 and p50 are reported to be cytosolic and, as shown above, to contain myristic acid. This suggested that it should also be possible to detect, by conventional cell fractionation procedures, soluble forms of p60^{src} which contain myristic acid. This experiment is difficult to perform

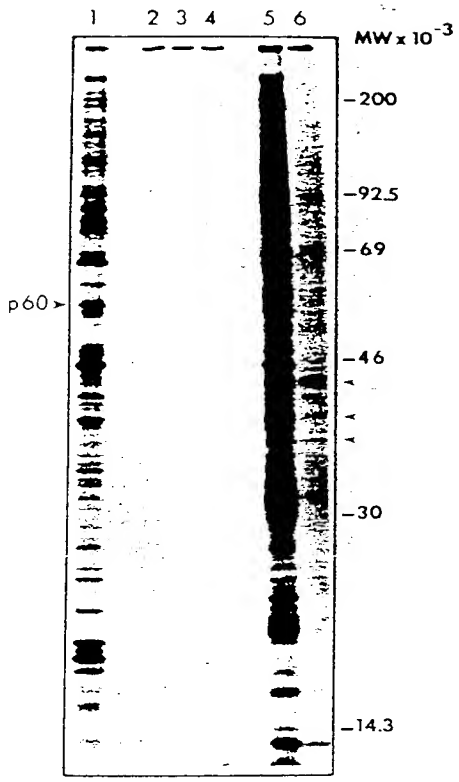

FIG. 2. Effect of cycloheximide on incorporation of [^{3}H]myristic acid and [^{3}H]palmitate into cellular proteins. SR-RSV-D-infected chicken cells were pretreated for the indicated times with 20 μg of cycloheximide per ml and then labeled in the continued presence of cycloheximide for 2 h with either [^{3}H]myristic acid or [^{3}H]palmitic acid. Cells were lysed directly in electrophoresis sample buffer, and samples containing 10^5 cells were analyzed by SDS-PAGE. Fluorographic exposure was for 14 days. p60^{src} is marked by an arrow, and the three proteins whose labeling with [^{3}H]palmitic acid was unaffected by cycloheximide are indicated. Lanes: 1, [^{3}H]myristic acid-labeled cells with no cycloheximide; 2, [^{3}H]myristic acid-labeled cells with cycloheximide and [^{3}H]myristic acid added simultaneously; 3, [^{3}H]myristic acid-labeled cells with 5 min of pretreatment with cycloheximide; 4, [^{3}H]myristic acid-labeled cells with 20 min of pretreatment with cycloheximide; 5, [^{3}H]palmitic acid-labeled cells with no cycloheximide; 6, [^{3}H]palmitic acid-labeled cells with 20 min of pretreatment with cycloheximide.

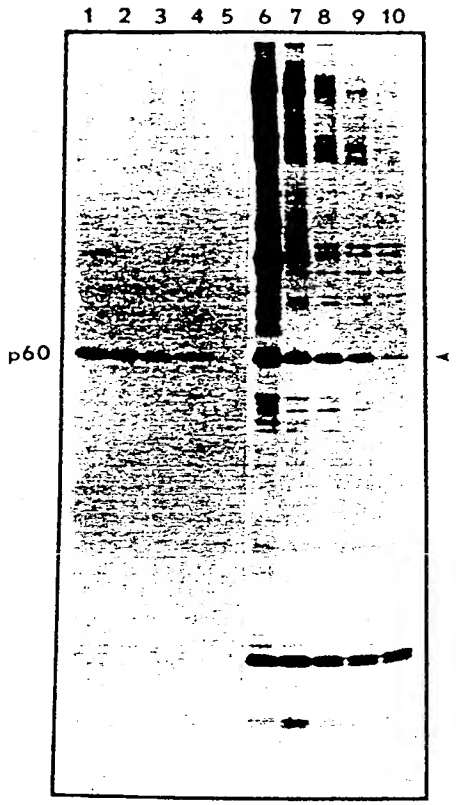

FIG. 3. Comparison of the turnover of p60^{src} labeled with [^{3}H]myristic acid or [^{35}S]methionine. SR-RSV-D-infected chicken cells were labeled for 2 h with [^{3}H]myristic acid or [^{35}S]methionine and then chased for 3 or 6 h in nonradioactive medium containing 0.18 mg of myristic acid (P-L Biochemicals, Inc.) per ml. Nonradioactive medium without the added myristic acid was then added to the plates used for the 10- and 22-h time points to prevent excessive accumulation of fatty acid. p60^{src} was immunoprecipitated with an excess of rabbit antitumor serum and analyzed by SDS-PAGE. The number of cells in both [^{3}H]myristic acid- and [^{35}S]methionine-labeled cultures doubled during the 24-h chase. The tracks containing the [^{3}H]myristic acid-labeled proteins were exposed for 5 days. The tracks containing the [^{35}S]methionine-labeled proteins were exposed for 1 day. Lanes 1, cells labeled with [^{3}H]myristic acid for 2 h; 2, [^{3}H]myristic acid-labeled cells with a 3-h chase; 3, [^{3}H]myristic acid-labeled cells with a 6-h chase; 4, [^{3}H]myristic acid-labeled cells with a 10-h chase; 5, [^{3}H]myristic acid-labeled cells with a 22-h chase; 6, cells labeled with [^{35}S]methionine for 2 h; 7, [^{35}S]methionine-labeled cells with a 3 h chase; 8, [^{35}S]methionine labeled cells with a 6-h chase; 9, [^{35}S]methionine-labeled cells with a 10-h chase; 10, [^{35}S]methionine-labeled cells with a 22-h chase.

chicken cells were labeled with [^{3}H]myristic acid and lysed in hypotonic buffer, and p60^{src} was isolated from particulate and soluble fractions generated by high-speed centrifugation. [^{3}H]myristate-labeled cell lysates were examined directly to ensure analysis of the entire population of p60^{src}. Thirty-six percent of the [^{3}H]myristic acid-containing p60^{src} molecules were found in the cytosol (Fig. 4). This demonstrates, in a more traditional manner, that myristic acid-modified p60^{src} can be cytosolic. The solubility of myristylated p60^{src} is not a unique property of the p60^{src} proteins encoded by temperature-sensitive mutant viruses. We also

resolved by SDS-PAGE. All of these myristylated proteins were present in the particulate fraction. Strikingly, at least

half could also be detected in the cytosolic fraction, and almost a third of the proteins were in fact more abundant in the soluble fraction than in the particulate fraction. Myristylated proteins are therefore not restricted to cellular membranes.

Myristylation of mutant p60^{src} is not temperature sensitive.

know is incorporated into pro[illegible]ons after catabolism of [^{3}H]myristic acid (Buss and Sefton, in press). We have now

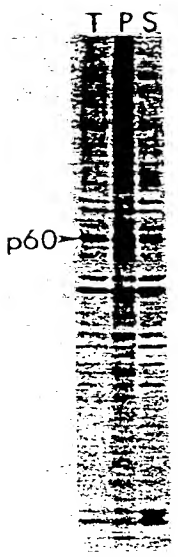

FIG. 4. [^{3}H]myristic acid-labeled proteins in soluble and particulate fractions. Chicken cells transformed by *ts*LA29 and grown at the permissive temperature (35°C) were labeled for 2 h with [^{3}H]myristic acid and homogenized and fractionated as described in the text. Fluorographic exposure was for 30 days. p60^{src} is indicated with an arrow. Lanes: T, unfractionated cell lysate; P, particulate fraction; S, soluble proteins.

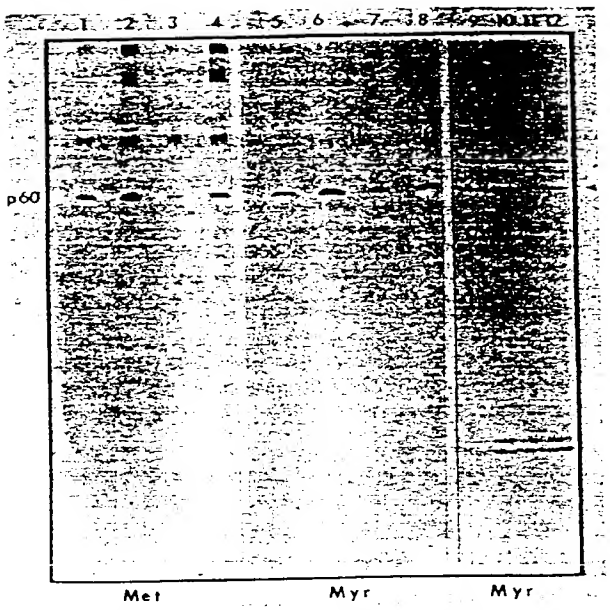

FIG. 5. [^{3}H]myristic acid incorporation into p60^{src} of *ts*NY68. Chicken cells infected with *ts*NY68 or SR-RSV-A were grown at 35 or 41°C as described in the text and labeled for 2 h with [^{3}H]myristic acid or [^{35}S]methionine. Immunoprecipitates were formed with antitumor serum and were resuspended in 50 μl of electrophoresis sample buffer. Proteins contained in 5 μl of [^{35}S]methionine-labeled immunoprecipitates or in 20 μl of [^{3}H]myristic acid-labeled immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. Exposure for the [^{35}S]methionine-labeled proteins was 1 day. Exposure of the immunoprecipitated [^{3}H]myristic acid-labeled proteins was for 1 day. The [^{3}H]myristic acid-labeled lysates were exposed for 5 days. Lanes: 1, [^{35}S]methionine-labeled cells, *ts*NY68, 35°C; 2, [^{35}S]methionine-labeled cells, *ts*NY68, 41°C; 3, [^{35}S]methionine-labeled cells, SR-RSV-A, 35°C; 4, [^{35}S]methionine-labeled cells, SR-RSV-A, 41°C; 5, [^{3}H]myristic acid-labeled cells, *ts*NY68, 35°C; 6, [^{3}H]myristic acid-labeled cells, *ts*NY68, 41°C; 7, [^{3}H]myristic acid-labeled cells, SR-RSV-A, 35°C; 8, [^{3}H]myristic acid-labeled cells, SR-RSV-A, 41°C; 9, total lysate from [^{3}H]myristic acid-labeled cells, *ts*NY68, 35°C; 10, total lysate from [^{3}H]myristic acid-labeled cells, *ts*NY68, 41°C; 11, total lysate from [^{3}H]myristic acid-labeled cells, SR-RSV-A, 35°C; 12, total lysate from [^{3}H]myristic acid-labeled cells, SR-RSV-A, 41°C.

examined whether the labeling of the mutant protein with [^{3}H]myristic acid was affected by temperature. Chicken cells infected with either *ts*NY68 or the parental wild-type virus SR-RSV-A were labeled for 2 h with [^{35}S]methionine or [^{3}H]myristic acid, and p60^{src} was isolated by immunoprecipitation with an excess of antitumor serum (Fig. 5). Because myristic acid appears to be added to p60^{src} immediately after the protein is synthesized (see above), comparison of the [^{35}S]methionine and [^{3}H]myristic acid incorporated into p60^{src} during this time will reveal accurately the extent to which p60^{src} becomes myristylated at the two temperatures. Because of the different growth rates of transformed and nontransformed cells and of cells grown at 35 and 41°C, the number of cells and therefore the amount of p60^{src} in the various cultures differed. The amount of [^{3}H]myristic acid-labeled p60^{src}, however, varied in parallel with the amount of [^{35}S]methionine-labeled p60^{src} (Table 1). The relative incorporation of [^{3}H]myristic acid into the mutant p60^{src} was not significantly different at 35 or 41°C and was, if anything, greater at 41 than at 35°C. The efficient incorporation of [^{3}H]myristic acid into the mutant p60^{src} at both 35 and 41°C was also apparent when [^{3}H]myristic acid-labeled lysates were analyzed directly (Fig. 5). The presence of [^{3}H]myristic acid in p60^{src} at the nonpermissive temperature was not due to leakiness of the mutant-infected cells. The *ts*NY68-infected cultures were completely transformed at 35°C but were morphologically normal at 41°C. Analysis of rates of 2-

parent, PR-RSV-A, at both the permissive and restrictive temperatures. When labeled with [^{3}H]palmitic acid for 2 h,

the amount of lipid in p60^{src} from either virus was slightly greater at 41 than at 35°C (Table 1).

It was possible that the 2-h labeling period failed to reveal steady-state differences in acylation of the mutant p60^{src} at 35 and 41°C. We have examined this question by labeling *ts*NY68-infected cells for 18 h with [^{3}H]palmitic acid. Here too we could detect no decrease in the lipid content of the p60^{src} protein of *ts*NY68 at the nonpermissive temperature (Table 1).

DISCUSSION

We have found here that myristic acid is added to p60^{src} in the cytosol before the deposition of the protein in mem-

with input and p60, the two cellular proteins, which mediate the movement of the protein to the plasma mem

TABLE 1. Lack of effect of temperature on incorporation of lipid into temperature-sensitive forms of $p60^{src}$

Virus	Temp (°C)	Labeling time (h)	Incorporation of:					
			[^{3}H]myristic acid (cpm)[a]	[^{35}S]methionine (cpm)[a]	Myr/Met[b]	[^{3}H]palmitic acid (peak area)[c]	[^{35}S]methionine (peak area)[c]	Palm/Met[b]
*ts*NY68	35	2	294	272	1.08			
	41	2	596	452	1.32			
SR-RSV-A	35	2	125	81	1.54			
	41	2	271	292	0.94			
*ts*LA29	35	2				37	376	0.10
	41	2				65	484	0.13
PR-RSV-A	35	2				13	146	0.09
	41	2				29	224	0.13
*ts*NY68	35	18				16	77	0.21
	41	18				36	125	0.29
SR-RSV-A	35	18				37	36	1.04
	41	18				65	86	0.76

[a] $p60^{src}$-containing pieces of the 2,5-diphenyloxazole-impregnated polyacrylamide gel (Fig. 5) were counted directly in scintillation fluid. A background of 30 cpm has been subtracted.

[b] Myr/Met, [^{3}H]myristic acid/[^{35}S]methionine; Palm/Met, [^{3}H]palmitic acid/[^{35}S]methionine.

[c] Arbitrary units from densitometric scans of fluorograms similar to Fig. 5.

brane (4, 8), already contains myristic acid. Somewhat similar observations have also been made by Cross and colleagues (10).

Although we have not yet determined whether myristic acid is added during or immediately after the synthesis of the protein, we favor the idea that myristylation is a cotranslational process, as is the case for the acetylation of amino termini (11, 23, 28), and that the nascent polypeptide chain is the substrate of the myristyl transferase. It is quite possible, in fact, that nascent chains are the only substrates of N-terminal myristyl transferases. Inhibition of protein synthesis with cycloheximide halted the incorporation of myristic acid completely. This suggested that myristic acid groups do not undergo turnover. Pulse-chase analysis demonstrated directly that this is indeed the case for $p60^{src}$. We suggest that myristylation is a permanent form of protein modification which occurs as the nascent polypeptide emerges from the polysome.

We initially suggested that lipid was added to $p60^{src}$ to convert the protein from a soluble species to a membrane protein. Since it now appears that the addition of lipid to $p60^{src}$ precedes the interaction of the protein with the plasma membrane, this idea, at least in its simplest form, is clearly incomplete. What then is the function of the myristyl moiety in $p60^{src}$? There is still good reason to think that it may help anchor the protein to cellular membranes. Cross et al. (10) and Garber and colleagues (13) have shown that a number of variants of $p60^{src}$ which do not contain myristic acid bind poorly to membranes and also do not cause cellular transformation. This suggests that the presence of myristate may in fact be essential for membrane binding and that the presence of the myristyl moiety in $p60^{src}$ may be indispensible for transformation. Nevertheless, the presence of myristic acid

group then does not make $p60^{src}$ insoluble in the cytoplasm.

The existence of soluble forms of $p60^{src}$ which contain myristic acid was not unanticipated. A number of other cellular proteins which contain myristic acid, including the catalytic subunit of the cyclic AMP-dependent protein kinase and calcineurin B, are found predominantly in the cytosol (Fig. 4; 6, 18). Myristylated proteins, including $p60^{src}$, thus are not invariably attached to the lipid bilayer. The myristyl group may have an additional function, quite unrelated to increasing the affinity of a protein for a lipid bilayer.

It is now clear that eucaryotic proteins undergo two very different forms of fatty acylation. Myristic acid is linked through an amide bond to the alpha-amino group of an amino-terminal glycine residue of $p60^{src}$ and a small number of other proteins (1, 6, 34). The myristylation of N-termini appears to occur very early in the life of a protein and may well take place before completion of the synthesis of the polypeptide. In the case of $p60^{src}$, the myristyl group appears to be metabolically stable and not to undergo turnover. The enzyme(s) which attaches fatty acids to amino-terminal glycines in general exhibits a strict specificity for myristic acid. Even when [^{3}H]palmitic acid is used to label $p60^{src}$, all of the inefficient labeling which is observed (39; Buss and Sefton, in press) reflects incorporation of [^{3}H]myristic acid arising from metabolism of the [^{3}H]palmitic acid (Buss and Sefton, in press). Furthermore, where chemical analysis has been possible, all of the fatty acid in the catalytic subunit, $p15^{gag}$, and calcineurin has been found to be myristic acid (1, 6, 15).

The modification of proteins with palmitic acid is a quite different process. Palmitic acid is not known to modify the amino termini of proteins. Rather it is often linked through alkali-labile bonds to internal amino acids within domains

of this carboxy-terminal tail of the protein (50). Palmitic acid appears to be attached to proteins only after their

release from the polysome. Several viral glycoproteins acquire palmitate in the Golgi apparatus after an obligatory lag of 20 min (33). Palmitylation is also clearly posttranslational for $p21^{ras}$ and the transferrin receptor (25, 39), because it is present only in the processed forms of these proteins. The enzyme(s) which palmitylates proteins also appears to utilize palmitic acid with some specificity. Chemical analysis of the G protein of vesicular stomatitis virus revealed that >80% of the fatty acid present was palmitate (32). Finally, palmitate is not necessarily a permanent protein modification. The palmityl moiety present in the transferrin receptor has been shown to undergo removal and replacement several times during the lifespan of the protein (25).

We have not been able to confirm that temperature-sensitive $p60^{src}$ proteins, which are largely cytosolic (12), contain a reduced amount of fatty acid at the nonpermissive temperature (13). We found that the acylation of the $p60^{src}$ proteins of both *ts*NY68 and *ts*LA29 viruses occurred equally well at 35 or 41°C. In addition, the abundance of fatty acid at steady state in the $p60^{src}$ protein of *ts*NY68 was identical at both the permissive and nonpermissive temperatures. It seems clear that the decreased ability of the mutant proteins to bind to cellular membranes at the restrictive temperature is not due to an absence of fatty acid and that the presence of myristic acid in $p60^{src}$ cannot be used as a means by which to distinguish soluble from membrane-bound forms of the enzyme.

There is recent evidence that $p60^{src}$ may phsophorylate phosphatidylinositol (40) as well as protein substrates. Since this phospholipid is confined to cellular membranes, binding to the plasma membrane could be important for the interaction of $p60^{src}$ with phosphatidylinositol. It is not unreasonable that the myristyl moiety in $p60^{src}$ plays a role in this interaction. Elucidation of the role of myristate in the several possible enzymatic activities of $p60^{src}$ should be facilitated greatly by site-directed mutations which prevent specifically the fatty acylation of the polypeptide.

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LITERATURE CITED

1. **Aitken, A., P. Cohen, S. Santikarn, D. H. Williams, A. G. Calder, A. Smith, and C. B. Klee.** 1982. Identification of the NH_2-terminal blocking group of calcineurin B as myristic acid. FEBS Lett. 150:314–318.
2. **Brugge, J., and D. Darrow.** 1982. Rous sarcoma virus-induced phosphorylation of a 50,000 molecular weight cellular protein. Nature (London) 295:250–253.
3. **Brugge, J., E. Erikson, and R. L. Erikson.** 1981. The specific interaction of the Rous sarcoma virus transforming protein, $pp60^{src}$, and two cellular proteins. Cell 25:363–372.
4. **Brugge, J., W. Yonemoto, and D. Darrow.** 1983. Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of this complex. Mol. Cell. Biol. 3:9–19.
5. [illegible] group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. Proc. Natl. Acad. Sci. U.S.A. 79:6128–6131.
7. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus transforming protein, $pp60^{src}$, shows protein kinase activity specific for tyrosine. Nature (London) 285:167–169.
8. **Courtneidge, S. A., and J. M. Bishop.** 1982. Transit of $pp60^{v\text{-}src}$ to the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. 79:7117–7121.
9. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{proto\text{-}src}$) are associated with the membrane. Proc. Natl. Acad. Sci. U.S.A. 77:3783–3787.
10. **Cross, F. R., E. A. Garber, D. Pellman, H. Hanafusa.** 1984. A short sequence in the $p60^{src}$ N-terminus is required for $p60^{src}$ myristylation and membrane association and for cell transformation. Mol. Cell. Biol. 4:1834–1842.
11. **Filner, B., and A. Marcus.** 1974. TMV coat protein synthesis in vivo: analysis of the N-terminal acetylation. Virology 61:537–546.
12. **Garber, E. A., J. G. Krueger, H. Hanafusa, and A. R. Goldberg.** 1983. Temperature-sensitive membrane association of $pp60^{src}$ in tsNY68-infected cells correlates with increased tyrosine phosphorylation of membrane-associated proteins. Virology 126:73–86.
13. **Garber, E. A., J. G. Krueger, H. Hanafusa, and A. R. Goldberg.** 1983. Only membrane-associated RSV *src* proteins have amino-terminally bound lipid. Nature (London) 302:161–163.
14. **Gilmore, T. D., K. Radke, and G. S. Martin.** 1982. Tyrosine phosphorylation of a 50K cellular polypeptide associated with the Rous sarcoma virus transforming protein $pp60^{src}$. Mol. Cell. Biol. 2:199–206.
15. **Henderson, L. E., H. C. Krutzsch, and S. Oroszlan.** 1983. Myristyl amino terminal acylation of murine retroviral proteins: an unusual post-translational protein modification. Proc. Natl. Acad. Sci. U.S.A. 80:339–343.
16. **Hunter, T., and B. M. Sefton.** 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. 77:1311–1315.
17. **Kawai, S., and H. Hanafusa.** 1971. The effects of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma virus mutant. Virology 46:470–479.
18. **Klee, C. B., and M. H. Krinks.** 1978. Purification of cyclic 3′,5′-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to Sepharose. Biochemistry 17:120–126.
19. **Kreuger, J. G., E. Wang, and A. R. Goldberg.** 1980. Evidence that the *src* gene product of Rous sarcoma virus is membrane-associated. Virology 101:25–40.
20. **Krzyzek, R. A., R. L. Mitchell, A. F. Lau, and A. J. Faras.** 1980. Association of $pp60^{src}$ and *src* protein kinase activity with the plasma membrane of nonpermissive and permissive avian sarcoma virus-infected cells. J. Virol. 36:805–815.
21. **Lee, J. S., H. E. Varmus, and J. M. Bishop.** 1979. Virus-specific messenger RNAs in permissive cells infected by avian sarcoma virus. J. Biol. Chem. 254:8015–8022.
22. **Levinson, A. D., H. Oppermann, H. E. Varmus, and J. M. Bishop.** 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. 255:11973–11980.
23. **Liew, C. C., G. W. Haslett, and V. G. Allfrey.** 1970. *N*-Acetylseryl-tRNA and polypeptide chain initiation during histone biosynthesis. Nature (London) 226:414–417.
24. **Omary, M. B., and I. S. Trowbridge.** 1981. Covalent binding of fatty acid to the transferrin receptor in cultured human cells. J. Biol. Chem. 256:4715–4718.
Omary, M. B., and I. S. Trowbridge. 1981. Biosynthesis of the [illegible] Sci. U.S.A. 78:1067–1071.
27. **Oppermann, H., L. Levintow, A. D. Levinson, H. E. Varmus,**

J. M. Bishop, and S. Kawai. 1981. Two cellular proteins that immunoprecipitate with the transforming protein of Rous sarcoma virus. Virology **113**:736–751.

28. **Pestana, A., and H. C. Pitot.** 1975. Acetylation of nascent polypeptide chains on rat liver polyribosomes in vivo and in vitro. Biochemistry **14**:1404–1412.
29. **Purchio, A. F., S. Jovanovich, and R. L. Erikson.** 1980. Sites of synthesis of viral proteins in avian sarcoma virus-infected chicken cells. J. Virol. 35:629–636.
30. **Rose, J. K., G. A. Adams, and C. J. Gallione.** 1984. The presence of cysteine in the cytoplasmic domain of the vesicular stomatitis virus glycoprotein is required for palmitate addition. Proc. Natl. Acad. Sci. U.S.A. **81**:2050–2054.
31. **Schmidt, M. F. G., M. Bracha, and M. J. Schlesinger.** 1979. Evidence for covalent attachment of fatty acids to Sindbis virus glycoproteins. Proc. Natl. Acad. Sci. U.S.A. **76**:1687–1691.
32. **Schmidt, M. F. G., and M. J. Schlesinger.** 1979. Fatty acid binding to vesicular stomatitis virus glycoprotein: a new type of post-translational modification of the viral glycoprotein. Cell **17**:813–819.
33. **Schmidt, M. F. G., and M. J. Schlesinger.** 1980. Relation of fatty acid attachment to the translation and maturation of vesicular stomatitis and Sindbis virus membrane glycoproteins. J. Biol. Chem. 255:3334–3339.
34. **Schultz, A., and S. Oroszlan.** 1984. Myristylation of *gag-onc* fusion proteins in mammalian transforming retroviruses. Virology 133:431–437.
35. **Schultz, A. M., and S. Oroszlan.** 1983. In vitro modification of retroviral *gag* gene-encoded polyproteins by myristic acid. J. Virol. **46**:355–361.
36. **Sefton, B. M., K. Beemon, and T. Hunter.** 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vitro and in vivo. J. Virol. **28**:957–971.
37. **Sefton, B. M., T. Hunter, and K. Beemon.** 1980. Temperature-sensitive transformation by Rous sarcoma virus and temperature-sensitive protein kinase activity. J. Virol. 33:220–229.
38. **Sefton, B. M., T. Patschinsky, C. Berdot, T. Hunter, and T. Elliott.** 1982. Phosphorylation and metabolism of the transforming protein of Rous sarcoma virus. J. Virol. **41**:813–820.
39. **Sefton, B. M., I. S. Trowbridge, J. A. Cooper, and E. M. Scolnick.** 1982. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. Cell **31**:465–474.
40. **Sugimoto, Y., M. Whitman, L. C. Cantley, and R. L. Erikson.** 1984. Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. Proc. Natl. Acad. Sci. U.S.A. **81**:2117–2121.
41. **Voronova, A. F., J. E. Buss, T. Patschinsky, T. Hunter, and B. M. Sefton.** 1984. Characterization of the protein apparently responsible for the elevated tyrosine protein kinase activity in LSTRA cells. Mol. Cell. Biol. **4**:2705–2713.
42. **Wyke, J. A., and M. Linial.** 1973. Temperature-sensitive avian sarcoma viruses: a physiological comparison of twenty mutants. Virology 53:152–161.